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FINE-TUNING OF THE SIGNALLING NETWORK CONTROLLING MORPHOGENESIS AND STEM CELL DEVELOPMENT IN TEETH

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Academic dissertation

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II Wang XP, **Suomalainen M**, Jorgez CJ, Matzuk MM, Werner S, Thesleff I. (2004) Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. Dev Cell 7:719-730.

III Wang XP*, **Suomalainen M***, Felszeghy S, Zelarayan LC, Alonso MT, Plikus MV, Maas RL, Chuong CM, Schimmang T, Thesleff I. (2007) An integrated gene regulatory network controls stem cell proliferation in teeth. PLoS Biol 5:e159. *Equal contribution

IV **Suomalainen M** and Thesleff I. (2009) Patterns of Wnt pathway activity in the mouse incisor indicate absence of Wnt/β-catenin signaling in the epithelial stem cells. Dev Dyn Epublished ahead of print Oct 5.

ABBREVIATIONS

ActR	Activin receptor
AI	Amelogenesis imperfecta
Alk	Activin-like kinase
APC	Adenomatous polyposis coli
BMP	Bone morphogenetic protein
co-Smad	Common mediator Smad
Ctip2	Chicken ovalbumin upstream promoter transcription factor-interacting protein 2
DFPC	Dental follicle precursor cell
Dll	Delta-like
Dkk	Dickkopf
DPSC	Dental pulp stem cell
E	Embryonic day
EDA	Ectodysplasin
ERM	Epithelial cell rests of Malassez
FGF	Fibroblast growth factor
Fst	Follistatin
HH	Hedgehog
HERS	Hertwig's epithelial root sheath
HSPG	Heparin sulfate proteoglycans
I-Smad	Inhibitory Smad
iPS cell	Induced pluripotent stem cell
K14	Keratin 14
LEF	Lymphoid enhancer factor
Lgr5	Leucine rich repeat containing G-protein coupled receptor 5
LRC	Label retaining cell
LRP	Lipoprotein receptor related protein
mRNA	Messenger RNA
miRNA	Micro RNA
MSX	Muscle segment homeobox gene
NICD	Notch intracellular domain
P	Post natal day
PAX	Paired box –containing transcription factor
PCP	Planar cell polarity
PDL	Periodontal ligaments
PDLSC	Periodontal ligament stem cell
PITX	Paired-like homeodomain
PTC	Patched
R-Smad	Receptor activated Smad
Smad	Homolog for *Drosophila* gene mother against decapentaplegic
SCAP	Apical papilla stem cell
SHED	Stem cells from human exfoliated deciduous teeth
SHH	Sonic hedgehog
Spry	Sprouty
TCF	T-cell specific transcription factor
TGFβ	Transforming growth factor β
Wnt	Wnt- family member

Gene names are written in italics and protein names in non-italics. Mouse genes or proteins are in lower case letters and human in upper case letter.

SUMMARY

Tooth development is regulated by sequential and reciprocal interactions between epithelium and mesenchyme. The molecular mechanisms underlying this regulation are conserved and most of the participating molecules belong to several signalling families. Research focusing on mouse teeth has uncovered many aspects of tooth development, including molecular and evolutionary specifics, and in addition offered a valuable system to analyse the regulation of epithelial stem cells. In mice the spatial and temporal regulation of cell differentiation and the mechanisms of patterning during development can be analysed both *in vivo* and *in vitro*.

Follistatin (Fst), a negative regulator of TGFβ superfamily signalling, is an important inhibitor during embryonic development. We showed the necessity of modulation of TGFβ signalling by Fst in three different regulatory steps during tooth development. First we showed that tinkering with the level of TGFβ signalling by Fst may cause variation in the molar cusp patterning and crown morphogenesis. Second, our results indicated that in the continuously growing mouse incisors asymmetric expression of *Fst* is responsible for the labial-lingual patterning of ameloblast differentiation and enamel formation. Two TGFβ superfamily signals, BMP and Activin, are required for proper ameloblast differentiation and Fst modulates their effects. Third, we identified a complex signalling network regulating the maintenance and proliferation of epithelial stem cells in the incisor, and showed that Fst is an essential modulator of this regulation. FGF3 in cooperation with FGF10 stimulates proliferation of epithelial stem cells and transit amplifying cells in the labial cervical loop. BMP4 represses *Fgf3* expression whereas Activin inhibits the repressive effect of BMP4 on the labial side. Thus, Fst inhibits Activin rather than BMP4 in the cervical loop area and limits the proliferation of lingual epithelium, thereby causing the asymmetric maintenance and proliferation of epithelial stem cells. In addition, we detected Lgr5, a Wnt target gene and an epithelial stem cell marker in the intestine, in the putative epithelial stem cells of the incisor, suggesting that Lgr5 is a marker of incisor stem cells but is not regulated by Wnt/β-catenin signalling in the incisor. Thus the epithelial stem cells in the incisor may not be directly regulated by Wnt/β-catenin signalling.

In conclusion, we showed in the mouse incisors that modulating the balance between inductive and inhibitory signals constitutes a key mechanism regulating the epithelial stem cells and ameloblast differentiation. Furthermore, we found additional support for the location of the putative epithelial stem cells and for the stemness of these cells. In the mouse molar we showed the necessity of fine-tuning the signalling in the regulation of the crown morphogenesis, and that altering the levels of an inhibitor can cause variation in the crown patterning.

REVIEW OF THE LITERATURE

1. Ectodermal organ development in the vertebrates

Vertebrates have many different ectodermal organs, such as teeth, hair, feathers, scales and beaks. Many exocrine glands, such as mammary and sweat glands, are also ectodermal organs. The similarities between different ectodermal organs are mostly visible in the placode and bud stage, but when the morphogenesis begins the similarities diminish. The interactions between epithelium and mesenchyme initiate the development. The first signal inducing differentiation comes from the mesenchyme in all ectodermal organs except the tooth where the signal originates in the epithelium (Pispa and Thesleff, 2003). After the initiation step the interaction between epithelium and mesenchyme is reciprocal and continuous, leading to complex structures of functional organs.

The first sign of ectodermal organ development is the thickening of the epithelium. Thickening forms a placode, a signalling centre, beneath which the mesenchymal cells condense. The forming structure begins to bud into or out of the mesenchyme, as the epithelium folds and branches at the end constructing the final structures of the organ. Regular arrangement of placodes, visible especially in the hair and feather follicle formation, is controlled by lateral inhibition as the concentrations of placode activating and inhibiting signals specify the location and formation of new placodes (Plikus and Chuong, 2008). The placode forms within a region where the level of activators is high and exceeds the effects of the inhibitor according to reaction diffusion model (Schlake and Sick, 2007). In tooth development the location of dental placodes are suggested to be determined by a balance between Fibroblast growth factor (FGF) and Bone morphogenetic protein (BMP) (Neübuser et al., 1997; Mandker and Neübuser, 2001).

Most of the organs are maintained by tissue turnover, repair and renewal throughout life. Self-renewing stem cells are responsible for this maintenance, thus they are distributed in almost every tissue. Stem cells have the ability to go through many cell divisions in an undifferentiated state and also the capacity to give rise to differentiated cell types, supplying new cells to the renewing tissue. Tissue turnover, for instance in the skin, can occur constantly, emphasizing the importance of the regulation of balance between proliferating and degradating cells. When a tissue is injured the healing processes, such as wound healing and scar formation, are initiated immediately in order to repair the tissue. Some organs renew cyclically, for instance hairs and feathers. In amphibians even whole complex organs, for instance limbs, can be regenerated.

2. Morphology and mechanisms of tooth development

Teeth are typical organs for the vertebrates, although in some species they have been lost during the evolution. The essential steps in tooth development include formation of the placode, budding of the epithelium into the underlying mesenchyme, condensation of the mesenchyme interacting with the bud, and folding and growth of the epithelium, which will finally create the shape of the tooth crown (Thesleff, 2006).

In many animals new teeth develop throughout life. In mammals teeth develop only once, as in rodents, or twice, as in

humans, forming primary and secondary dentition. Recently several researchers have sought to find a suitable model organism to study the formation of the secondary dentition, for instance ferrets, and enlighten the mechanisms of the regulation behind the secondary dentition (Järvinen et al., 2008, 2009). Fish, like other non-mammalian vertebrates, have continuous renewal and replacement of teeth, thus creating another kind of model extending understanding of control of the tooth development (Stock, 2007). Birds, on the other hand, are an example of toothless vertebrates. They lost their teeth over 80 million years ago but the genes regulating the tooth development still exist in the jaws, and transplanted mouse neural crest derived cells can induce tooth development in the chicken (Francis-West et al., 1998; Schneider et al., 1999; Mitsiadis et al., 2003, 2006).

Rodents have two kinds of teeth, molars and incisors, separated by a toothless area, the diastema. Thus rodents lack both the canines and premolars, which are typical for many other mammals. While premolar development occurs during the early stages of development, it is arrested at the bud stage and the bud is removed apoptotically (Keränen et al., 1999; Peterkova et al., 2002). Some mutant mouse lines have an extra tooth in front of the first molar, in the area of prospective premolars. For instance overexpressing *Ectodysplasin* (*Eda*) under *Keratin14* promoter (*K14-Eda*) or disrupting balance of FGF signalling by knocking down *Sprouty2* (*Spry2*) or *Spry4* expression leads to the formation of extra molar within the diastema (Mustonen et al., 2003; Klein et al., 2006).

The tooth can be structurally divided to a crown and a root. The morphology of the crown is strictly regulated during the development, leading to complex cusp patterning especially in the molars. The cusps form as a result of folding of the dental epithelium, which is regulated by enamel knots, the epithelial signalling centres (Jernvall et al., 1994; Vaahtokari et al., 1996). However, the teeth in fish and reptiles are mostly conical and lack cusps, and mammalian incisors and canines also lack the multicuspal structure. The morphological differences between incisors and molars become evident during the late stages of development.

The patterning of the molar crown, i.e. the regulation of the cusp formation, is strictly regulated by activators and inhibitors. According to the morphodynamic model activators induce the formation of an enamel knot, whereas inhibitors prevent the differentiation of the enamel knot, instead promoting growth (Salazar-Ciudad and Jernvall, 2002). The morphodynamic model predicts concentration peaks of activators and inhibitors which correspond to the gene expression patterns detected in the teeth. Suggested activator factors are BMPs, and inhibiting factors include FGFs and sonic hedgehog (Shh) (Salazar-Ciudad and Jernvall, 2002).

The tooth crown is covered with enamel and dentin. Mesenchymal cells adjacent to the inner dental epithelium begin to differentiate into dentin forming odontoblasts, and the remaining dental papilla cells form the dental pulp. Enamel depositing ameloblasts differentiate from the inner dental epithelium. Epithelium differentiates also into the stellate reticulum, surrounded by stratum intermedium and inner or outer dental epithelia. Ameloblast and odontoblast differentiation is regulated by interactions between epithelium and mesenchyme.

The completion of crown formation is an onset of root development. The epithelial cervical loops lose stellate reticulum and the remaining epithelium, comprising only from inner and outer dental epithelia and the basal lamina, forms Hertwig's epithelial root sheath (HERS). HERS guides root development and induces the formation of dentin and cementum, formed by cementoblasts differentiating from dental follicle cells. Later the HERS fractures and forms epithelial cell rests of Malassez (ERM) (Nanci, 2008). Recent results suggest that in some exceptions HERS is not necessary for the formation of ERM, and regulation between crown and root fate is flexible (Tummers and Thesleff, 2008). In addition to the roots, other supporting tissues are needed for the firm attachment of the tooth. Periodontal ligaments (PDL), alveolar bone, gingiva and cementum play a role in tooth attachment. The dental follicle gives rise to fibroblasts which synthesize fibers and several other substances, eventually forming PDL. PDL are responsible for attaching the tooth to the alveolar bone, but they also act as sensory innervations (Nanci, 2008, pp. 261).

In several mammalian species, such as rabbits and rodents, certain teeth grow continuously. Mice have continuously growing asymmetrical conical incisors but certain sibling vole has also continuously growing molars (Tummers and Thesleff, 2003). The beginning of the growth and differentiation of the continuously growing molar and incisor resemble that of the root forming molar, but expression of several signalling molecules, which are lost when the root development begins in the molar, are maintained in continuously growing teeth (Tummers and Thesleff, 2003). However, the typical gene expression pattern for continuously growing teeth was maintained in the *in vitro* cultured mouse molar and it is suggested that epithelium of the molar still has the capacity to grow continuously depending on the regulation of surrounding environment (Tummers et al., 2007).

2.1 Initiation of tooth development

Tooth development is regulated by reciprocal and sequential interactions between the mesenchyme and epithelium. These interactions regulate proliferation, differentiation and apoptosis, and they are common among all organs formed from epithelium and mesenchyme, thus in most of the organs.

The dental lamina (also called odontogenic band), a horseshoe shaped thickening of the epithelium (Figure 1), forms from epithelial cells derived from the first branchial arch and is supported by underlying neural crest derived mesenchymal cells. Teeth develop only within the dental lamina, and even the development of the extra teeth, present in several different mutant mice, is restricted to the dental lamina. Although continuous activation of Wnt/β-catenin signaling in the oral epithelium induces ectopic teeth and continuous tooth formation in mice, the teeth in most of the mutants developed within the dental lamina (Järvinen et al., 2006; Kuraguchi et al., 2006; Liu et al., 2008). However, recent results in Adenomatous polyposis coli (Apc), a member of a protein complex targeting β-catenin to degradation, loss-of function mouse teeth indicated that in certain cases teeth can develop outside the lamina (Wang et al., 2009). Tooth placodes begin to form within the dental lamina at embryonic day (E) 11. The first signals to initiate the tooth development come from the epithelium (Mina and Kollar, 1987).

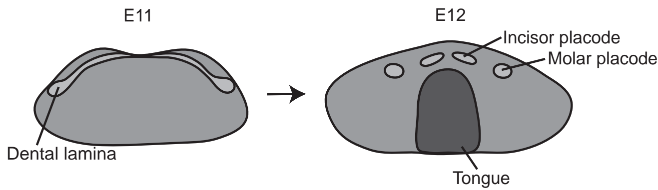

Figure 1.
The expression pattern of *Pitx2* indicating the dental lamina in a mouse mandible at E11 and E12. Figure drawn according to Maria Jussila's pictures.

Nevertheless, the mesenchyme also has to be competent to react to the signals. Later, after the initiation stage, the odontogenic potential shifts to the dental mesenchyme (Mina and Kollar, 1987).

3. Signalling pathways regulating ectodermal organ development

Interactions between cells and tissues can be transported through signalling pathways and cell-cell contacts. The signalling pathways consist of a signalling molecule, a receptor and usually an intracellular signalling cascade. The signalling molecules, ligands, are secreted from the cell or attached to the cell membrane. The ligands induce an activation of a receptor in a target cell. The activation of the receptor usually initiates an intracellular signalling cascade, including several modulators, which eventually leads to the regulation of a target gene. The ligand can be an autocrine factor and interact with the secretory cell itself or a paracrine factor and interact with neighbouring cells (Figure 2). When the signalling requires a cell-cell contact between signalling cells

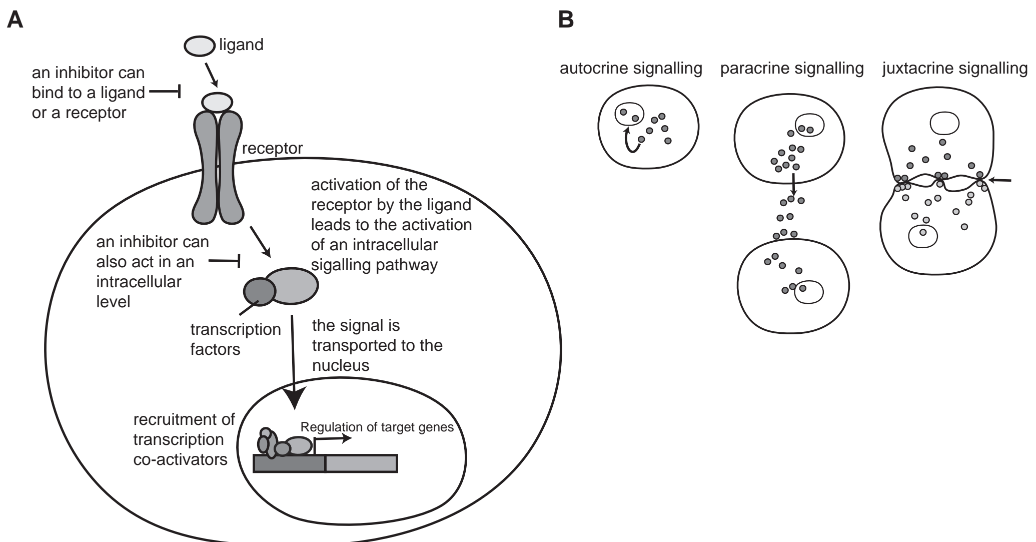

Figure 2.
A. A hypothetical signalling pathway showing different steps of a signalling cascade. A ligand binds to a receptor which leads to activation of intracellular signalling pathway finally regulating the target genes.
B. Different types of signalling; autocrine, paracrine and juxtacrine signalling. Autocrine signalling activates the secretory cell itself whereas paracrine signalling requires the secretion of signalling molecules from one cell to induce the activation in the neighbouring cell. Juxtacrine signalling requires cell-cell contacts between interacting cells.

the interaction is called juxtacrine. The signalling requires intensive control and fine-tuning because even small changes in signal activity can lead to drastic changes during development. Although the expression and function of ligands, receptors and intracellular signalling pathway modulators are tightly regulated, signalling still needs to be adjusted by inhibitors at various steps. Inhibitors can antagonise the signalling by inhibiting the functions of the ligand, the receptor, the intracellular pathway or the gene expression in the nucleus. The inhibitors bind specifically to their target molecules, and they are introduced later in detail together with the pathways they interact with.

Another mechanism of regulation, microRNAs (miRNAs), has also been shown to participate in fine-tuning of the signalling. miRNAs are conserved and abundant class of small RNAs that do not code proteins, but instead function as regulatory molecules silencing their target genes at the posttranscriptional level (Bartel, 2009). In mammals miRNAs function usually by inhibiting translation by pairing to their target mRNAs but they can also degrade mRNAs (Bartel, 2004). miRNAs are involved in many processes, for example stem cell differentiation and in cell fate decision (Houbaviy et al., 2003; Hatfield et al., 2005; Karp and Ambros, 2005; Zhang et al., 2007; Jevnaker and Osmundsen, 2008).

The genes regulating development in different organs are conserved among vertebrates. The expression of genes is regulated spatially and temporally. The initiation and development of ectodermal derived organs is triggered by the temporal recruitment of highly conserved signalling molecules such as Transforming growth factor β (TGFβ), FGF, Wnt, SHH, Notch and Eda signalling. All of these signalling pathways are activated by secreted ligands except Notch pathway, in which ligands are bound to the cell membrane. The signalling pathways are complex, including transmembrane receptors and usually several different intracellular signalling pathways.

3.1 TGFβ superfamily

The TGFβ superfamily, including BMPs, TGFβs, Activins and Inhibins, has important roles in patterning of the embryo and tissue homeostasis, as well as in controlling many different functions such as proliferation, differentiation and apoptosis of the cells (Balemans and van Hul 2002; Kitisin et al., 2007; Wu and Hill, 2009). During the early gastrulation TGFβ family ligands have an essential role determining the dorsoventral pattern of the mesoderm. BMPs and Activins are important molecules in axes formation and patterning during the early stages of development. They operate as morphogens, forming concentration gradients and regulating development in a dose-dependent manner (Wu and Hill, 2009). The TGFβ superfamily signalling pathways include heteromeric receptors, several different intracellular pathways, Smad complexes, and proteins interacting with receptors or Smads. Signalling pathways are even regulated by many extracellular or intracellular inhibitors, such as Follistatin (Fst), Noggin, Sostdc1 (also called Ectodin), Chordin, Cerberus, Smurf and BAMBI (Wu and Hill, 2009). The functions of inhibitors are in many occasions redundant, for instance Fst, Noggin, and Chordin have to be simultaneously depleted in *Xenopus* embryo to cause the loss of embryonic dorsalisation, whereas single mutations

cause only moderate changes (Khokha et al., 2005).

3.1.1 Receptors and intracellular signalling pathways

The TGFβ superfamily receptors are serine – threonine kinase transmembrane receptors formed from two subunits, type I and II receptors (Wu and Hill, 2009). TGFβ ligand binds to the type II receptor which then phosphorylates the type I receptor. The phosphorylation of type I receptor activates the kinase activity leading to the activation of intracellular downstream pathway and determining the specificity of intracellular signal (Figure 3). Several different downstream pathways are known, of these Smad dependent pathway is the best characterized. The TGFβ pathway can also signal at least through ERK and JNK pathways (Derynck and Zhang, 2003; Miyazono et al., 2005).

Four different type II receptors and seven type I receptors have been identified (Kitisin et al., 2007). The specificity of the intracellular signals is principally determined by type I receptors (Miyazono et al., 2005). A phosphorylated type I receptor activates the intracellular signalling cascade by phosphorylating Smads (Figure 3) (Derynck and Zhang, 2003; Feng and Derynck, 2005). There are three different kinds of Smad transcription factors: receptor activated

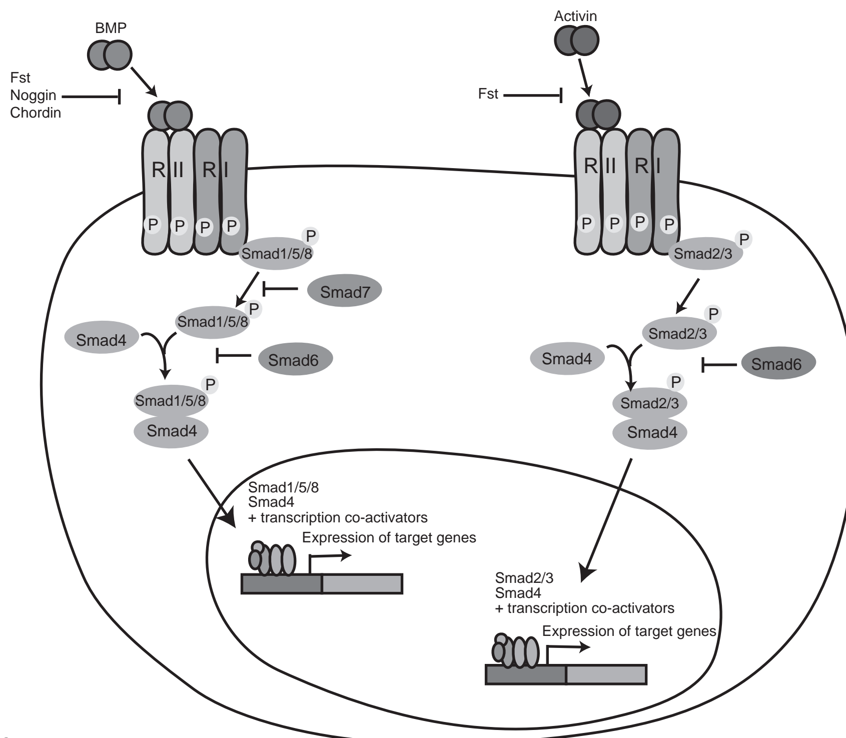

Figure 3.
BMP and Activin signalling pathways. In both pathways ligand binds to a type II receptor (RII) which phosphorylates a type I receptor (RI) leading to the activation of intracellular Smad signalling. BMP signalling activates R-Smads 2 and 3 whereas Activin signalling activates R-Smads 1, 5 and 8. Co-Smad, Smad4, can form a complex with R-Smads from both pathways. Complete Smad complex is translocated into the nucleus where it regulates transcription. Smad6 and Smad7 antagonise the intracellular signalling by inhibiting the activation of R-Smads and Smad4.

Smads (R-Smads), common mediator Smads (co-Smads) and inhibitory Smads (I-Smads). R-Smads are specific for BMP, Activin or TGFβ signalling. Receptor activated Smad1, Smad5 and Smad8 are phosphorylated by BMPs and Smad2 and Smad3 are activated by Activin (Feng and Derynck, 2005). The phosphorylated type I receptor phosphorylates R-Smad, which is then released and moves to interact with co-Smad. In mammals there is only one co-Smad, Smad4, which, after forming a complex with two R-Smads, translocates to the nucleus and regulates transcription (Nohe et al., 2004). Inhibitory Smad6 and Smad7 negatively regulate signalling of TGFβ superfamily members by preventing the activation of R-Smad and co-Smad (Feng and Derynck, 2005).

TGFβ superfamily members have many different inhibitors. Negative regulation of BMP signalling during the early embryogenesis is essential for development of dorsal-ventral axis and induction of nervous system formation. Inhibitors of BMPs are expressed by the organiser of the gastrulation during *Xenopus* development, and include Noggin, Chordin, Nodal-related protein 3 and Fst (Gilbert, 2006, pp. 328). Variability between TGFβ superfamily inhibitors is created through different binding properties. For instance Fst has higher affinity in binding to Activin than to BMPs, but Noggin specifically binds and inhibits only BMPs (Nohe et al., 2004, Harrison et al., 2004).

3.1.2 BMPs

BMPs were first identified as molecules stimulating cartilage and bone formation, and indeed they participate in most of the processes associated with skeletal morphogenesis. Later BMPs were found to also have a significant role in embryonic development and essential regulatory functions in many occasions, for instance in tissue homeostasis (Nohe et al., 2004; Gilbert, 2006, pp. 153). In early embryogenesis BMP4 functions as a morphogen regulating other genes in a concentration dependent manner and is an important ventraliser of the tissues in addition to functioning in left-right axis formation. BMPs induce ectoderm to become epidermal, and in order to enable nervous system to differentiate from the ectoderm BMPs need to be inhibited. During early gastrulation, a cocktail of inhibitors secreted by the node regulate BMPs. In addition, BMPs induce somite development and they are important in patterning, for instance anterior-posterior axis of digits. In several tissues, including teeth, BMPs function as inhibitors of differentiation, and instead promote growth.

Although there are more than 20 different BMPs, only three type I and type II receptors are known to mediate BMP signalling (Nohe et al., 2004; Nie et al., 2006; Kitisin et al., 2007). Various *Bmp* knockout mice have emphasized the essential role of BMP signalling in the development. For instance, *Bmp2*$^{-/-}$ and *Bmp4*$^{-/-}$ mice die during early stages of embryogenesis, and *Bmp7*$^{-/-}$ are perinatally lethal (Winnier et al., 1995; Zhang and Bradley 1996; Luo et al., 1995; Dudley et al., 1995).

3.1.3 Activin

Activins are morphogens and important regulators during early embryogenesis (Green and Smith, 1990). Activin induces mesodermal tissues, for instance development of muscles and notochord, and depending on the Activin gradient genes regulate development of different mesodermal tissues in *Xenopus* embryo

(Smith et al., 1990; van den Eijnden-Van Raaij et al., 1990). Activins also participates in cell fate determination and left-right axis formation by inhibiting *Shh* expression and activating *Fgf8* expression on the right side of the embryo. Activins have also other important functions in tissue growth, differentiation and maintenance. Activins are secreted dimers, consisting of different subunits (Sulyok et al., 2004; McDowall et al., 2008). Mice deficient in ActivinβA have severe defects in the craniofacial region and die within 24 hours of birth (Matzuk et al., 1995a). In contrast, mice deficient in ActivinβB are viable but have defects in eyelid development and reproduction (Schrewe et al., 1994; Vassalli et al., 1994). Mice homozygous to both ActivinβA and ActivinβB had combinatorial phenotypes of single Activin mutants but no additional defects, indicating that ActivinβA and ActivinβB are not functionally redundant (Matzuk et al., 1995a). Activin induces the expression of Fst, the inhibitor of both Activin and BMPs, thus forming a negative feedback loop leading to tightly controlled Activin expression (Michel et al., 1993; Hemmati-Brivanlou et al., 1994; De Winter et al., 1996; Iemura et al., 1998).

3.1.4 Follistatin - inhibitor of Activin and BMPs

Fst is the most studied Activin inhibitor (Nakamura et al., 1990; Patel, 1998; McDowall et al., 2008). Fst also inhibits BMPs and is an important negative regulator already during early embryogenesis (Michel et al., 1993; De Winter et al., 1996; Iemura et al., 1998; Fainsod et al., 1997). In *Xenopus* embryos Fst is an essential inhibitor of BMP during neural induction (Hemmati-Brivanlou et al., 1994). However, loss of Fst in mouse does not cause neural or mesodermal defects due to the redundant functions of other inhibitors (Matzuk et al., 1995b). Fst is a secreted molecule with different isoforms formed by alternative mRNA splicing at the 3’ end (Sugino et al., 1993). Activin binds to Fst with higher affinity than to Activin receptor, thus Fst is a powerful inhibitor of Activin (Schneyer et al., 1994; Thompson et al., 2005). Fst has two different mechanisms for inhibiting Activin, either by binding to Activin to inhibit binding between Activin and its receptor, or by binding to Activin on the membrane followed by internalization and degradation of the Fst-Activin complex (McDowall et al., 2008; deWinter 1996; Harrington et al., 2006). The inhibition of BMP occurs when Fst binds both the ligand and BMP type I receptor simultaneously (Iemura et al., 1998). Loss of *Fst* causes serious developmental problems, resulting in perinatal lethality in *Fst*$^{-/-}$ mice due to lung problems (Matzuk et al., 1995b). In addition, *Fst*$^{-/-}$ mice have defects in the hard palate and in the skin, the growth is retarded, and the whiskers are abnormal (Matzuk et al., 1995b). Overexpression of *Fst* under *Keratin14* promoter (*K14-Fst*) causes reduced body weight, larger ears and longer tails than in the wild type littermates, and wound healing is also severely delayed (Wankell et al., 2001).

3.2 FGF pathway

FGFs induce many different responses including proliferation, differentiation, migration and inhibition of apoptosis (Bates, 2006). Over 20 FGF ligands have been found in vertebrates and their functions are adjusted by several inhibitors and feedback loops (Katoh and Katoh, 2006; Orniz and Itoh, 2001). The structure of FGFs in conserved among

the vertebrates and they all share a high affinity for heparin sulfate proteoglycans (HSPG). Heparin and HPSG interact with ligand and receptor to enhance the formation of the complex (Ornitz and Itoh, 2001).

FGF receptors (FGFr) are single transmembrane tyrosine kinase receptors. They can be tissue specific, either expressed in the epithelium, as FGFr1IIIb and FGFr2IIIb, or in the mesenchyme as FGFr1IIIc and FGFr3 (Kettunen et al., 1998). Hitherto four different genes encoding FGFr have been identified, and three of these can produce multiple receptor isoforms through alternative splicing (Bates, 2006). The receptors form from two subunits which are induced to dimerize upon ligand binding leading to autophosphorylation and activation of the intracellular pathways (Thisse and Thisse, 2005). FGFs signal through three different intracellular pathways, of which the best characterised is Ras-ERK MAP kinase (Itoh, 2007).

One level of FGF signalling regulation occurs through Sproutys (Spry) which inhibit the receptor tyrosine kinases interacting with Ras-ERK MAP kinase signalling (Mason et al., 2006). *Sprys* are induced by FGFs, thus interactions result a negative feedback loop. According to the current view Sprys need to be bound to the membrane in order to function (Kim and Bar-Sagi, 2004). Out of four known Sprys three have been detected in the developing tooth (Klein et al., 2006).

The phenotypes of various *Fgf* knockouts vary from embryonic lethality to minor changes in adults. *Fgf4*$^{-/-}$ mice are early embryonically lethal, and *Fgf9*$^{-/-}$ and *Fgf10*$^{-/-}$ mice die shortly after birth (Colvin et al., 2001; Feldman et al., 1995; Min et al., 1998; Sekine et al., 1999). *Fgf3*$^{-/-}$ mice are viable with mild defects in the inner ear and some skeletal problems (Alvarez et al., 2003). In many cases FGFs are functionally redundant (Ornitz and Itoh, 2001).

3.3 Wnt pathway

In early *Xenopus* embryogenesis Wnt signalling participates in inducing muscle formation, specification of neural cells, generating polarity, and it regulates dorsal axis. Wnt signalling pathway has been indicated to participate in cell fate specification, proliferation, differentiation and it has been connected with and extensively studied in the context of cancer and stem cells (Chu et al., 2004; Gordon and Nusse, 2006; Blanpain et al., 2007; Haegebarth and Clevers, 2009). Until now, 19 Wnt ligands have been described in mammals.

Wnt pathway consists of several different intracellular signalling pathways activated by Wnt ligands, including Wnt/β-catenin pathway and several pathways previously referred as non-canonical pathways including planar cell polarity (PCP), Ca^{2+} related pathways and signalling mediated through receptors Ror2 or Ryk (Gordon and Nusse, 2006). Several pathways share molecules (He et al., 1997; Holmen et al., 2002; Liu et al., 2005; Mikels and Nusse, 2006). Complexity of signalling is also formed when same ligands can activate different intracellular pathways depending on the receptors and the identity of the cell receiving the signal, leading to a situation where the pathways are not easy to distinguish (Clevers, 2006; van Amerongen et al. 2008; Gordon and Nusse, 2006). Earlier the ligands were thought to regulate which intracellular pathway was activated. For instance Wnt5a was first identified as non-canonical ligand, but recently it was

shown to activate or inhibit Wnt/β-catenin pathway depending on the context (Mikels and Nusse, 2006).

The best characterized pathway for the present is the Wnt/β-catenin pathway (Figure 4). The activation of the Wnt/β-catenin pathway occurs when the Wnt ligand binds to Frizzled, a transmembrane receptor, and a lipoprotein receptor related protein (LRP) 5/6 co-receptor activates the interaction between receptor complex and intracellular Dishevelled. This is followed by the inactivation of the degradation protein complex and accumulation of β-catenin in the nucleus where it interacts with members of Lymphoid enhancer factor (LEF)/ T cell factor (TCF) family of transcription factors and activates gene expression (Clevers, 2006). When Wnt ligands are absent and the pathway is inactive, β-catenin is phosphorylated and targeted to degradation by a protein complex consisting of several molecules including Axin, APC, CK1, and Glycogen synthase kinase 3 (GSK3).

Less characterized pathways include PCP, Ca^{2+} related pathways and signalling mediated through receptors Ror2 or Ryk (van Amerongen et al., 2008). Wnt/PCP and Wnt/Ca^{2+} pathways are mostly known to participate in the regulation of cell movements and orientation (Moon et al., 1993; Ungar et al., 1995; Slusarski et al., 1997; Jaffe, 1999). Activation of Wnt/PCP pathway leads to Dishevelled mediated activation of c-jun N-terminal kinase

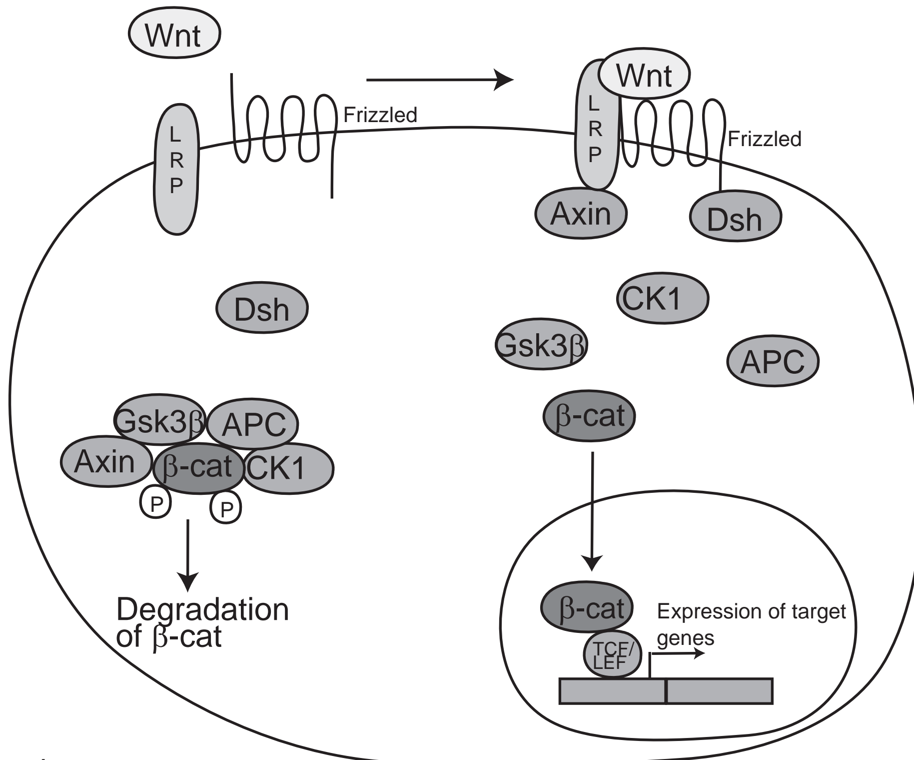

Figure 4.
Wnt/β-catenin signaling pathway is activated when β-catenin binds to Frizzled receptor and together with LRP5/6 co-receptor activates Dishevelled (Dsh). This leads to the disintegration of cytoplasmic destruction complex, thus stabilized β-catenin accumulates to the nucleus and activates its target genes.

(JNK) which affects the cytoskeletal dynamics (Yamanaka et al., 2002). Also Frizzled has a role in Wnt/PCP pathway. However, although at least several essential molecules participate both in Wnt/β-catenin and Wnt/PCP pathway, the pathways are considered to be separate and several key Wnt/β-catenin pathway molecules, such as LRP, TCF and β-catenin do not participate in Wnt/PCP pathway (van Amerongen et al., 2008). Wnt binding to Wnt/Ca^{2+} signalling pathway leads also to Frizzled mediated activation of intracellular signalling, the release of Ca^{2+} in the cells and the activation of Ca^{2+} dependent molecules (Slusarski et a., 1997; Kühl et al., 2000). There is some controversy over whether Wnt ligands are always or at all needed for the activation of Wnt/Ca^{2+} pathway, and the same question has also arisen concerning the Wnt/PCP pathway activation (van Amerongen et al., 2008; Seifert and Mlodzik, 2007; Hendrickx and Leyns, 2008). Two new receptors have recently been connected with Wnt signalling, an atypical tyrosine kinase Ryk and receptor tyrosine kinase Ror2. Ryk and Ror2 have Wnt binding domains and they can interact at least with Wnt1, Wnt3a and Wnt5a (Kikuchi et al., 2007; Nusse, 2008)

The Wnt pathway is closely regulated by several inhibitors. Dickkopf1 (Dkk1), Dkk2 and Dkk4 antagonise Wnt signalling, but the role of the fourth Dkk family molecule, Dkk3, remains controversial (Caricasole et al. 2003; Hoang et al. 2004; Niehrs, 2006; Fliniaux et al., 2008; Lee et al. 2008). Dkks inhibit Wnt signalling by binding to LRP6 and preventing Wnt from binding to the receptor. As co-receptor Kremen binds to Dkk the receptor complex is internalized through endocytosis (Niehrs, 2006). Sostdc1 inhibits Wnt signalling by binding to LRP6 (Itasaki et al., 2003). Cerberus, Wnt inhibitory factor (Wif) and Secreted Frizzled related protein (Sfrp) family molecules are also Wnt inhibitors (Hsieh et al., 1999; Jones and Jomary, 2002; Kawano and Krypta, 2003).

The Wnt signalling pathway has many different mouse models, and knock out mice have been constructed of almost all Wnt ligands. The phenotypes are often severe and in many cases cause massive defects in early gastrulation, defects in axis formation for instance (Liu et al., 1999). In humans heterozygotic loss of *Wnt10a* or *Axin2* causes ectodermal defects and colorectal cancer (Adaimy et al., 2007; Lammi et al., 2004).

3.4 Notch pathway

Notch pathway can activate or inhibit many different regulatory events such as cell-fate termination, boundary formation, stem cell maintenance and cell proliferation, and it is often connected with inhibition of cell differentiation (High and Epstein, 2007). An important function of Notch signalling during embryonic cell-fate termination is lateral inhibition, i.e. the mechanism how Notch can cause two identical cells to adopt different fates (Doe and Goodman, 1985). In mammals, the Notch pathway consists of five ligands and four receptors. The ligands belong to two different families, Jagged and Delta-like (Dll). Although Dll3 is structurally similar to other Dlls it is unclear whether Dll3 functions as Notch ligand (Chiba, 2006). The ligands are transmembrane proteins, thus they only activate adjacent cells.

The Notch receptor is activated following ligand binding and leads to consecutive cleavages of the receptor and the cleavage of Notch intracellular domain (NICD) by γ-secretase. NICD is then

transferred into the nucleus where it forms a complex with co-activators leading to the activation of the transcription (Fortini, 2009). Recent results also suggest that cellular context has an influence on the amplitude and duration of Notch signalling activity (Kopan and Ilagan, 2009).

Notch signalling can be modulated by several molecules acting either antagonistically or synergistically depending on the cellular and receptor context. Fringe proteins can enhance or inhibit signalling, and Numb, which often antagonise Notch signalling, can in some cases function also synergistically with Notch (Kopan and Ilagan, 2009).

Most of the mouse mutants with a gene knocked out from the Notch signalling family have phenotype indicating that not much redundancy exists between Notch pathway genes. Mice deficient in a single Notch pathway gene can either have minor phenotype defects or die during the development. For instance mice deficient in Notch1, Notch2, Dll1 or Jagged1 are embryonically lethal, but Jagged2 deficient mice die perinatally (Swiatek et al., 1994; Conlon et al., 1995; Hamada et al., 1999; Xue et al., 1999; Jiang et al., 1998). Notch3 or Notch4 knock out mice, however, are viable and fertile (Krebs et al., 2000, 2003).

3.5 Shh pathway

The Hedgehog (Hh) family consists of three known molecules, Desert hedgehog (Dhh), Indian hedgehog (Ihh), and Sonic hedgehog (Shh). Hedgehog molecules have essential roles in left-right asymmetry, formation of the organs, in patterning of the limbs and in stem cell maintenance. During development Hh molecules act as gradient depending morphogens controlling distinct cell fates (Ingham and McMahon, 2001).

Shh is the only Hh molecule expressed in the developing tooth (Bitgood and McMahon, 1995). Shh binds to the multipass transmembrane receptor Patched (Ptc) thereby releasing another multipass-transmembrane protein, Smoothened, from the suppression of Ptc. This leads to the activation of the intracellular signalling cascade through serine threonine kinase 3 (STK3) and stabilisation of Gli family members (Katoh and Katoh, 2006). Intracellular signalling is mediated by Gli transcription factors and the end result can be either activation or inhibition of the target gene. In the absence of Ptc activation, Glis are ubiqutinated and partially degraded.

3.6 Ectodysplasin pathway

The Ectodysplasin (Eda) pathway is one of the Tumor Necrosis Factor (TNF) superfamily members. Eda is essential for the development of many ectodermally derived organs, and defects in its signalling pathway causes a syndrome called hypohidrotic ectodermal dysplasia (HED) in humans. Eda pathway is activated when the Eda ligand binds to a Eda receptor (Edar) and modulates the cytosolic adaptor molecule Edar-associated death domain (Edaradd) (Mikkola and Thesleff, 2003). The binding of Edar to Edaradd leads to interaction with other adaptor molecules, for example Traf6 *in vivo*. This complex activates an intracellular signalling cascade leading to the activation of the canonical Nuclear factor-κB (NF-κB) pathway (Mikkola, 2009). Eda has several known target genes. For instance, it regulates the expression of two BMP antagonists, connective tissue growth factor (Ccn2/ctgf) and Fst, as well as Dkk4, a Wnt

inhibitor (Pummila et al., 2007; Fliniaux et al., 2008).

Mouse models deficient in Eda, Edar or Edaradd arose by spontaneous mutations, but the genetic basis of the pathway was discovered only several years ago (Kere et al., 1996; Srivastava et al., 1997; Yan et al., 2000; Headon et al., 2001). The phenotypes of these mouse mutants are similar and malformations can be seen for instance as reduced number of hairs, missing and misshapen teeth and the total absence of sweat glands (Mikkola et al., 2009). In humans HED causes phenotypic characteristics resembling that of the mouse phenotype. Eda has been shown to promote placodal cell fate but it is not the first signal to trigger fate decision (Mustonen et al., 2004).

4. Molecular regulation of tooth development

4.1 Initiation and tooth identity

BMPs and FGFs, expressed in the epithelium, are essential participants in the initiation of tooth development. FGF8 induces mesenchymal expression of transcription factor *Pax9* in the branchial arch mesenchyme at E10, while BMP4 inhibits *Pax9* expression (Neubüser et al., 1997). In *Pax9*$^{-/-}$ mice, tooth development arrests at the bud stage, due to the lack of mesenchymal expression of *Bmp4*, *Msx1* and *Lef1*, regulated by Pax9 (Peters et al., 1998). *Pitx2* and *Shh* are first expressed restrictedly in the dental lamina and later in the tooth placodes (Keränen et al., 1999). During the initiation of tooth development the odontogenic potential is situated in the epithelium, thus the first signal comes from the epithelium and regulates the mesenchyme. However, the odontogenic potential remains in the epithelium only for a short while, and at E12.5 the potential shifts to the mesenchyme, which then becomes the regulator of further tooth development (Kollar and Baird 1970; Mina & Kollar 1987; Vainio et al., 1993; Chen et al., 1996). After the shift the mesenchyme is also capable of inducing nondental epithelium to develop and form a tooth (Kollar & Baird1969; Ruch1987; Vainio et al., 1993). The formation of epithelial thickenings, or placodes, within the dental lamina, indicates the beginning of the tooth development at E11. Signalling centers within the placodes express many molecules regulating tooth development and induce condensation in the underlying mesenchyme (Åberg et al., 1997; Dassule and McMahon, 1998; Keränen et al., 1998; Sarkar and Sharpe, 1999). The epithelial placodes express *Bmp4*, and BMP4 induces the expression of several genes, including *Msx1*, *Msx2*, *Bmp4* and *Lef1*, in the condensing mesenchyme (Vainio et al., 1993; Kratochwil et al., 1996; Bei and Maas, 1998). Deficiency in several different genes for instance both *Msx1* and *Msx2*, *Pitx2* or *p63* arrests tooth development in the initiation stage, (Lin et al., 1999; Satokata et al., 2000; Laurikkala et al., 2006). Schematic presentation of molar and incisor development is in Figure 5.

4.2 Bud stage

The tooth bud begins to form as an invagination of the epithelium at E11.5. The epithelial cell population at the tip of the bud forms a new signalling centre at the late bud stage, the primary enamel knot, which begins to express wide variety of signalling molecules belonging to many different signalling families, including *Fgf*s, *Bmp*s, *Shh*, and *Wnt*s (Thesleff, 2003). The primary enamel knot,

consisting of condensed non-proliferating epithelial cells, is essential in regulating proliferation and the continuation of the tooth development, and it remains to direct the tooth development through the cap stage and regulate formation of secondary enamel knots (Jernvall et al., 1994). *Msx2* and *p21*, which are induced by BMP4 from the mesenchyme, are the early markers of the enamel knot (Jernvall et al., 1998). p21, a cyclin-dependent kinase inhibitor associated with cessation of proliferation and beginning of terminal differentiation, might be involved with the differentiation of enamel knot cells (Bloch-Zupan et al., 1998; Jernvall et al., 1998).

The transition stage between bud and cap stage is an important step triggering the development of the tooth crown morphology, and in many mice deficient in a certain gene tooth development is arrested at this step. For instance in *Fgfr2b*$^{-/-}$, *Pax9*$^{-/-}$, *Lef1*$^{-/-}$, *Msx1*$^{-/-}$ and *Bmpr1A*$^{-/-}$ mice tooth development is arrested at the bud stage (van Genderen et al., 1994; Chen et al., 1996; Peters et al., 1998; De Moerlooze et al., 2000). In *Activinβ A*$^{-/-}$ mouse the development of incisors and mandibular molars is arrested at the bud stage, but the maxillary molars are unaffected, indicating that some other signalling pathway must regulate the development of maxillary molars (Ferguson et al., 1998).

4.3 Cap stage and development of tooth crown morphology

The transition from the bud to cap stage begins as the bud starts to grow and surround condensed mesenchyme, the dental papilla. The tips of the flanking epithelium surrounding the dental papilla form cervical loops which later participate in root development. Development of the cervical loop, comprised of stellate reticulum, stratum intermedium and inner or outer dental epithelium, is initiated by the primary enamel knot, but while the flanking epithelium continues to grow

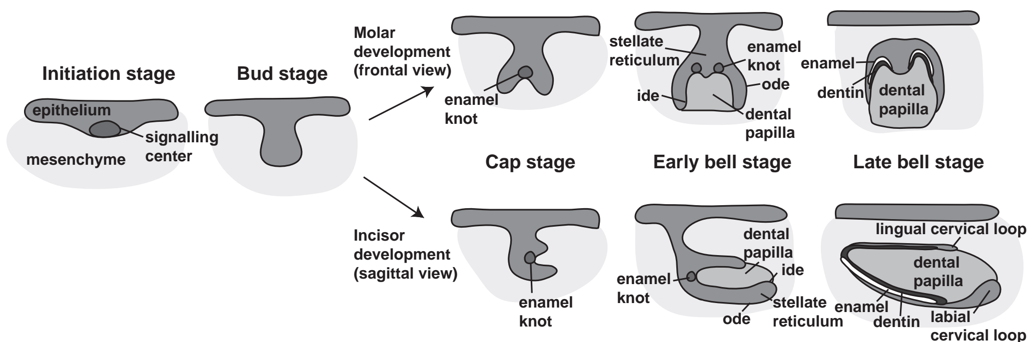

Figure 5.
Embryonic development of mouse teeth. The early stages of molar and incisor development are similar. The first signal of the tooth development comes from the epithelium and the early signalling centre begins to regulate the underlying mesenchyme. The differences between molar and incisor begin to arise during the cap stage and the characteristic shapes and structures of both teeth are distinct at the bell stage. Enamel knot, an epithelial signalling centre appears at the cap stage to regulate the tooth development. Epithelium is in dark grey and mesenchyme in light grey. Abbreviations: ide, inner dental epithelium; ode, outer dental epithelium.

the regulatory role of the enamel knot is lost. Typical characteristics of the cap stage, regulated by primary enamel knot, are rapid cell proliferation and folding of the epithelium, which lead to the changes in crown morphogenesis (Jernvall et al., 1994). The primary enamel knot is a transient structure and it is removed by BMP4 induced apoptosis during the transition from cap to bell stage (Vaahtokari et al., 1996; Jernvall et al., 1998). During the bell stage the shape of the crown is determined and the variable morphologies of the teeth begin to take shape under strict regulation. The primary enamel knot controls the localisation of secondary enamel knots, which arise in area of the future cusp tips and as signalling centres regulate the tooth morphogenesis and cusp patterning (Jernvall et al., 1994). According to the morphodynamic model, activators, presumably BMPs, and inhibitors, presumably FGFs and Shh, are expressed in the primary enamel knot and regulate the formation and localisation of secondary enamel knots (Salazar-Ciudad and Jernvall, 2002). The activator promotes cell differentiation by inducing the formation of an enamel knot, but the inhibitor promotes growth and inhibits differentiation. The dynamics of the activator and inhibitor regulate the area where enamel knots are formed and cell differentiation occurs. Tooth shape varies widely between different mammalian species, and even the different teeth within a species are variable. The variability between tooth morphologies is regulated by the same set of genes indicating the importance of fine tuning of the gene network. The secondary enamel knots express many signalling molecules, for instance *Fgf4*, *Fgf9*, *Shh*, *Bmp4*, *Wnt10b*, *p21*, *Lef1* and *Msx2* (Keränen et al., 1998; Kettunen and Thesleff, 1998).

4.4 Bell stage and cell differentiation

The differentiation of the distinct dental cell types, which is also regulated by epithelial mesenchymal interactions, begins at the early bell stage. Many aspects concerning the cell differentiation still remain unclear due to the complicated and reiterative nature of this signalling. During the bell stage odontoblasts and ameloblasts differentiate and begin to form dentin and enamel, covering the tooth crown. Secondary enamel knots disappear at the late bell stage after the cusps are initiated (Vaahtokari et al., 1996). *Slit3*, marker of primary and secondary enamel knots, remains in several cells of the secondary enamel knot after the histological structures of the knot are lost (Løes et al., 2001; Luukko et al., 2003). Later *Slit3* is detected in a cluster of epithelial cells remaining next to the enamel free area of the molar cusps, suggesting a presence of tertiary enamel knot (Luukko et al., 2003).

Odontoblasts differentiate from the dental papilla mesenchymal cells adjacent to the inner dental epithelium. The differentiation of the odontoblasts begins from the tips of the cusps next to the secondary enamel knots which presumably participate in odontoblast differentiation (Thesleff et al., 2001). The cells in a single cell layer aligning the inner dental epithelium first differentiate into cuboidal preodontoblasts, which elongate into columnar odontoblasts and become polarised. Shh is necessary for the organisation and polarisation of the odontoblast cell layer, although it is not needed for cellular differentiation (Dassule et al., 2000). Odontoblasts produce dentin matrix, consisting mainly of type I collagen. Mesenchymal pulp cells are able to maintain the dentin by differentiating

to odontoblasts and regenerating dentin in case of injury, for example caries (Nanci, 2008, pp.103, 205).

Several molecules have been shown to induce odontoblast differentiation *in vitro*, including members of the TGFβ signalling family and FGFs (Vaahtokari et al., 1991; Cam et al., 1992; Bègue-Kirn et al., 1992, 1994). A combination of either BMP2 and TGFβ1 or FGF1 and TGFβ1 induce the differentiation of odontoblasts in the isolated dental papilla (Bègue-Kirn et al., 1992; Unda et al., 2000). *Bmp4* is expressed in the preodontoblasts but it is downregulated in the odontoblasts where *Bmp2* expression, nonetheless, is upregulated (Nakashima 1994; Nakashima and Reddi, 2003, Yamashiro et al., 2003). BMP2 and BMP4 stimulate odontoblast differentiation *in vitro* (Vainio et al., 1993). These results suggest that BMPs have an essential role in the odontoblast differentiation. In addition, BMP2, BMP4 and BMP7 induce odontoblasts to produce dentin, and BMP2 recombinant protein induces differentiation of dental pulp stem cells into odontoblasts *in vitro*, indicating that BMPs also participate in the regeneration of odontoblasts (Nakashima, 1994; Rutherford et al., 1994; Iohara et al., 2004). Wnt signalling has also been indicated as a participant in dentin formation. Continuous stabilisation of β-catenin in the mesenchyme disrupts the differentiation of both odontoblasts and ameloblasts (Chen et al., 2009). In addition, *Wnt10a* is expressed in the secretory odontoblasts together with dentin sialophosphoprotein (Dspp), and it is suggested to be upstream regulator of Dspp, and to participate in dentin mineralization (Yamashiro et al., 2007).

Ameloblasts are columnar and polarised cells differentiating from the inner dental epithelium and forming an organised cell layer lining the basement membrane. Ameloblast differentiation can be divided in three consecutive stages, presecretory, secretory and maturation, which are responsible for different functions. The ameloblast layer is characterised by strong cell-cell adhesion, especially during secretory stage. Ameloblasts synthesise and secrete proteins specific to enamel, eventually forming enamel, the most highly mineralized tissue in the vertebrates. Unlike odontoblasts, ameloblasts are removed by apoptosis after maturation of the enamel or at the eruption of the tooth. Thus, after the enamel is formed it cannot be regenerated anymore. As the structure of the enamel is highly mineralized and organised, it partially compensates for this limitation on regeneration.

The differentiation of ameloblasts requires signals from the functional odontoblasts and predentin or dentin, but ameloblasts themselves also express many genes presumably participating in cell differentiation and enamel formation (Zeichner-David et al., 1995). Enamel secretion begins once dentin mineralization has started. To date several genes, most of them participating also in odontoblast differentiation, have been suggested to regulate ameloblast differentiation, for instance BMPs and FGFs. *Bmp2*, *Bmp4*, *Bmp5* and *Bmp7* are expressed in the epithelium and ameloblasts, suggesting a contribution to ameloblast differentiation (Åberg et al., 1997; Heikinheimo et al., 1998). Upregulation of BMP inhibitor *Noggin* under keratin14 (*K14-Noggin*) cause abnormal differentiation of ameloblasts and defective or lost enamel layer (Plikus et al., 2005). In addition, *Bmp2* and *Tgfβ1*, expressed by odontoblasts, induce ameloblast differentiation *in vitro* (Coin et al., 1999).

The role of FGFs is shown in the mouse incisor where unbalanced FGF signalling results ectopic ameloblast differentiation (Klein et al., 2008).

Amelogenesis imperfecta (AI) is a heterogenous group of inherited defects in enamel formation. The variability in the enamel defects in many cases is caused by the timing of amelogenesis disruption. Changes in several ameloblast specific genes, in Amelogenin, Ameloblastin, Enamelysin, Kallikrein-related peptidase-4 and Fam83H, are known to cause defects in the enamel, (Gibson et al., 2001; Caterina et al., 2002; Paine et al., 2003; Fukumoto et al., 2004; Hart et al., 2004; Kim et al., 2008). Similarly, many different genes have been reported to cause defects in the enamel in various mouse mutants, either in loss of function or gain of function situations. For instance, overexpression of *Eda*, *Wnt3* or *Tgfβ1* causes aberrant or lost enamel layer (Haryuama et al., 2006; Millar et al., 2003; Mustonen et al., 2004). *Shh*, expressed both in the preameloblasts and ameloblasts, has a role in ameloblast differentiation and proliferation, as shown through loss of function experiments (Dassule et al., 2000; Gritli-Linde et al., 2002). In *Msx2* deficient mice, ameloblasts reach secretory stage and the secreted enamel is abnormal, indicating that Msx2 controls ameloblast terminal differentiation (Bei et al., 2004). Mutations in Distal-less homeobox 3 (Dlx3) are associated with tricho-dento-osseous syndrome in humans causing for instance hypoplasia in the enamel (Price et al., 1998). Deficiency of *T-box 1* (*Tbx1*) causes loss of ameloblasts and enamel in mouse (Catón et al., 2009). Upregulated FGF signalling induces ectopic expression of *Tbx1* and *Amelogenin*, indicating the signalling interaction (Mitsiadis et al., 2008a; Catón et al., 2009). The phenotype in Chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (*Ctip2*, also called *Bcl11b*) deficient mice show unpolarised ameloblasts in the unorganised ameloblast cell layer, but surprisingly also ectopic ameloblasts in the lingual side of the incisor (Golonzhka et al., 2009).

4.5 Continuously growing mouse incisors

Mouse incisors grow continuously to replace the wear of the tooth on the apical end. The maintained asymmetrical cervical loops, which harbour epithelial stem cells, and asymmetric ameloblast differentiation and enamel deposition are two characteristics responsible for the distinct structures of incisor. The deposition of enamel only on the labial side, and thus lingual side covered predominantly with dentin, keeps the teeth sharp as the upper and lower incisors are gnawed against each others. However, due to the asymmetric enamel distribution incisors are also more susceptible to wear, and continuous regeneration is required for tooth maintenance. Due to the morphology the lingual side of the incisor is called the root analogue and the labial side, covered by enamel, is called the crown analogue. The molecular mechanisms regulating the formation of asymmetric structures of the incisor began to be uncovered in this thesis work.

The initial morphological differences between molar and continuously growing mouse incisors arise early in the cap stage. In the cap stage incisor the flanking epithelium, which is beginning to surround the condensed mesenchyme, starts to proliferate and grow asymmetrically, leading to different sized lingual and labial cervical loops (Figures 5 and 6). Labial

cervical loop cells proliferate efficiently and the labial epithelial tissue becomes larger than the lingual cervical loop. The cervical loop area consists of the loose stellate reticulum cells surrounded by a denser cell layer of stratum intermedium. The outermost epithelial cell layer is the basal layer, which on the tooth papilla side is called the inner dental epithelium and on the follicle side outer dental epithelium.

The differences between lingual and labial cervical loops of the incisor increase after cell differentiation begins. Odontoblast differentiation begins on the both sides, but ameloblast differentiation occurs only on the labial side (Figure 6). As development continues dentin is secreted around the incisor but enamel is restricted on the labial side. Ameloblasts in different consecutive stages can be visualised from the proximal to apical end on the labial side of an incisor, as newly differentiated, pre-secretory stage ameloblasts reside in the proximal area and the ameloblasts in the maturation stage reside in the apical area. During the late bell stage of molar development the stellate reticulum of the cervical loop is lost and the tissue begins to differentiate to the HERS. However, in the incisor the stellate reticulum is sustained, consisting of only a few cells on the lingual side but on the labial side harbouring large compartment of loose cells. The cervical loop comprises the epithelial stem cells of the incisor, which are responsible for continuous ameloblast differentiation and enamel formation (Harada et al., 1999). In the molar HERS initiates root formation and the fragments of HERS give rise to ERM. However, on the lingual side of the continuously growing mouse incisor, in the continuously growing roots of sloth molar and in *K14-Eda* mice incisors epithelium fragments and forms ERM although HERS is not formed, thus questioning whether HERS is always necessary for root formation (Tummers and Thesleff, 2008). I will discuss the molecular mechanisms regulating incisor development later.

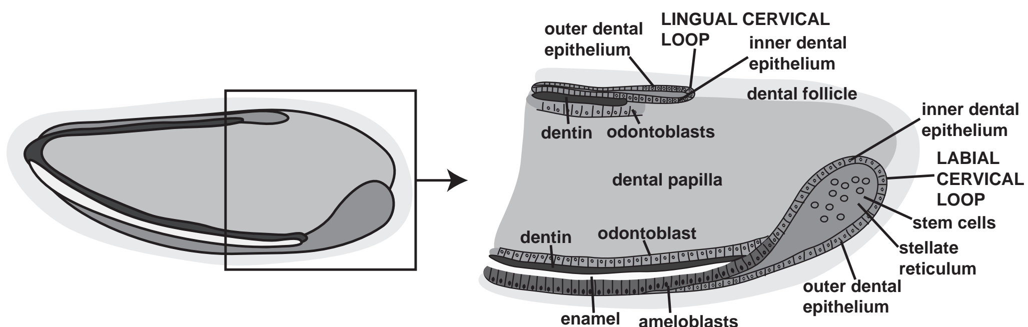

Figure 6.
The structure of a continuously growing mouse incisor during the late bell stage. Labial and lingual cervical loop have distinguished structures. Lingual cervical loop consists only of inner and outer dental epithelium whereas labial cervical loop includes additionally large stellate reticulum harbouring epithelial stem cells. Odontoblasts differentiate and dentin is formed on both labial and lingual side but ameloblast differentiation and enamel formation is restricted to the labial side.

5. Stem cells

In addition to their role in embryogenesis, stem cells are needed to maintain tissue homeostasis in normal cell turnover and in case of injury in the adult. Defining a stem cell is complicated due to different characters and variable amount of plasticity identified in undifferentiated cells. During early embryonic development cells can be totipotent and are able to differentiate into all kinds of cells of the body. Later stem cell plasticity diminishes and different characteristics between stem cells and differentiated cells become more ambiguous. A remarkable amount of plasticity remains in some adult stem cells, for instance a mammary gland or a crypt of the intestine can be rebuild from a single cell (Shackleton et al., 2006; Sato et al., 2009).

5.1 Characteristics of stem cells

Stem cells can be defined as self-renewing, slowly proliferating, having potency to differentiate into a single or several type of differentiated cells and forming more stem cells out of a single cell (Melton and Cowan, 2004; Slack, 2009). During proliferation the stem cell generates two daughter cells with similar characteristics to the stem cell or one daughter cell that is identical to the stem cell and another committed cell beginning to differentiate and thus having more restricted plasticity (Molofsky et al., 2004; Mitsiadis et al., 2007). The committed cell can give rise to increasingly committed cells with less plasticity and which might show some kind of differentiation. The differentiation capability of the stem cell diminishes gradually and it is regulated by the surrounding environment. Usually, committed cells cannot return to less committed stage, but sometimes differentiated cells can transdifferentiate into some other kind of cell type. During embryonic development transdifferentiation occurs, for example, in neural crest cells which are originally epithelial cells but transdifferentiate into mesenchymal cells before migrating to the branchial archs (Gilbert, 2006). Recently, induced pluripotent stem (iPS) cells were induced from embryonic and adult fibroblast cells, and they share many characteristics typical to embryonic stem cells, most importantly the potential to differentiate into tissues from all three germ layers (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Meissner et al., 2007; Wernig et al., 2007; Yamanaka et al., 2007). iPS cells may be in the future a source of stem cells for tissue engineering, substituting the embryonic stem cells and thus resolving ethical problems concerning the use of embryonic derived stem cells. The iPS cells are induced using Oct3/4, Sox2, c-myc and Klf4 (Takahashi and Yamanaka, 2006). Proto-oncogene c-myc is a probable cause of tumorigenic characteristics typical to some of the iPS cells, thus substituting molecules have been under search, the newest results showing that hypoxia may be a solution (Yoshida et al., 2009). Thus, many characteristics of iPS cells and the technique of cell induction still need to be analysed precisely before clinical applications can be tested.

Another typical character of stem cells, slow proliferation, is thought to occur to avoid accumulating mutations. In mammals stem cells are usually in a quiescent state or cell cycle is extremely slow (Cheshier et al., 1999; Melton and Cowan, 2004). However, recent results have indicated that stem cells are not always quiescent (Barker et al., 2007). Stem cells and the surrounding tissue together create a niche (Ohlstein et al.,

2004; Moore and Lemischka, 2006). The niche is often responsible for keeping the stem cells in an undifferentiated state and maintaining their stemness, thus inhibiting differentiation in an environment consisting of differentiated cells. Several molecules participating in the regulation of stem cells have been uncovered. For instance, addition of BMP to the serum *in vitro* induces downstream target Inhibitor of differentiation (Id), which maintains the undifferentiated state of the stem cells and inhibits neuronal differentiation (Ying et al., 2003). Also ActivinA is capable for maintaining the self-renewal and pluripotency of human embryonic stem cells *in vitro* by inducing several molecules including *Oct4*, *Nanog*, *Wnt3* and *Fgf8*, and suppressing BMP signal (Xiao et al., 2006).

Identifying a stem cell is complicated because common molecules for stem cells still remain unrecognised and may not even exist, and in many tissues a specific stem cell marker is unknown. Most known stem cell markers identify only one or few types of different stem cells. This suggests that stem cell niches in different tissues are regulated by variable signalling. One typical feature of stem cells, the slow cycling velocity, has been used as an identifying character. Long term label-retention method labels slowly cycling cells and it has been used to label stem cells for instance in the hair follicle and intestine (Cotsarelis et al., 1990; Potten et al., 1974). However, recently new results concerning the localisation of stem cells in the intestine and the validity of stem cell recognition by label retaining method have been questioned. The +4 cells in the intestine were recognized as stem cells because of their status as label retaining cells (LRC). Conversely to this, recent experiments, done with a stem cell marker, identified the stem cells among crypt base cells (Barker et al., 2007). These cells are actively proliferating cells, indicating that occasionally slow proliferation cannot be utilised as a character identifying stem cells. An overview of common or proposed stem cell markers in epidermis, feathers, hairs, intestine, bone marrow and mouse incisors is in Table1.

5.2 Stem cell niche

The current hypothesis is that the stem cells are located in particular stem cells niches in the tissues. The niche is a compartment that maintains stem cells in an undifferentiated stage and regulates proliferation (Melton and Cowan, 2004; Ohlstein et al., 2004; Moore and Lemischka, 2006; Mitsiadis et al., 2007; Fuchs, 2009). It consists of stem cells and the surrounding tissue, comprised of both differentiated cells and extracellular matrix (Tumbar et al., 2004). The combination of intrinsic stem cells factors and the niche maintains the undifferentiated stage of stem cells and regulates proliferation. The microenvironment surrounding the stem cells forms a barrier against differentiation inducing signalling and, simultaneously, the stem cells in the niche express genes that maintain the quiescence. When the stem cells are removed from the niche they show a high degree of plasticity by proliferating and differentiating actively, thus demonstrating the importance of the niche as a regulatory environment (Watt and Hogan, 2000). The stem cell niche is a unique environment which varies in different tissues, and in addition, the development of the stem cells and niches remains unknown. However, it is not evident that stem cells always need a niche. For instance, in the mammalian intestine stem cells are located between

Table1.
An overview of regulatory genes or signalling networks and proposed or common markers of several stem cells.

Stem cell tissue	Regulatory genes/ signalling networks of stem cells	Proposed/common marker genes	References for signalling networks	References for marker genes
Incisor epihtelium (cervical loop)	FGF10, Notch1, FGF3*, BMP4*, Activin*, Fst*, Spry, Shh	Identified as label retaining cells, Lgr5*	Harada el al., 1999; Dassule et al., 2000; Tummers and Thesleff, 2003; Klein et al., 2008	Harada et al., 1999
Tooth mesenchyme (papilla)	Notch, BMP2	Recognition with similar group of markers as bone marrow stem cells, single-colony selection, Notch1, Nestin	Mitsiadis et al., 1999; Iohara et al., 2004	Gronthos et al., 2000; Seo et al., 2004; Morsczeck et al., 2005a; Sonoyama et al., 2006
Hair follilce (bulge)	Wnt/β-catenin, BMP2, BMP4, TGFβ, Shh, Notch, FGFs, p63	Integrinα6, CD34, Lgr5 (during telogen), Keratin15, Keratin14, p63, Tcf3	van Genderen et al., 1994; Zhou et al., 1995; St-Jaques et al., 1998; Kratochwil et al., 1996; DasGupta and Fuchs, 1999; Blanpain et al., 2004; Kobielak et al., 2003, 2007; Andl et al., 2004; Vauclair et al., 2005	Li et al., 1998; Lyle et al., 1998; Mills et al., 1999; Yang et al., 1999; Kaur and Li, 2000; Liu et al., 2003; Trempus et al., 2003; Jaks et al., 2008
Epidermis	Notch, mitogen-activated protein kinase (MAPK), nuclear factor-κB, Wnt/β-catenin, BMP, Shh, Id, p63	Integrinβ1	Seitz et al., 1998; Fan and Khavari, 1999; Yang et al., 1999; Rangarajan et al., 2001; Nickoloff et al., 2000; Koster et al., 2004; Tumbar et al., 2004; Mou et al., 2006; Blanpain et al., 2006; Senoo et al., 2006	Barrandon and Green, 1987; Jones and Watt, 1993; Jones et al., 1995
Feather	Wnt1, Wnt3a, Wnt11, Dkk, Shh, BMP2, BMP4, Notch	Identified as label retaining cells	Jung et al., 1998; Viallet et al., 1998; Morgan et al., 1998; Scaal et al., 2002; Chang et al., 2003	Yue et al., 2005
Intestine (crypt)	Wnt/β-catenin signaling, BMP, PTEN, Notch	Lgr5, Prominin1, Bmi1	Korinek et al., 1998; Jensen et al., 2000; Pinto et al., 2003; Kuhnert et al., 2004; Ireland et al., 2004; Haramis et al., 2004; van Es and Clevers, 2005; Fevr et al., 2007; He et al., 2004, 2007	Barker et al., 2007; Sangiorgi and Capeachi, 2008; Montgomery and Shivdasani, 2009
Bone marrow	Bmi1, GATA2, Gfi1, Lmo2, Runx1, Scl/tal1, Tel, Mll, Jagged1, Notch, CXCR4	Recognition for example with high levels of c-Kit (CD117), Sca1, CD34 and CD150; low levels of CD244, CD48, B220, CD3, CD4, CD5, CD8, Mac1, GR1, Ter119 and NK1.1	Calvi et al., 2003; Sugiyama et al., 2006; Begley et al., 1989; Boehm et al., 1988; Miyoshi et al., 1991; Golub et al., 1995; Ellisen et al., 1991; Collins and Rabbitts, 2002; Lessard and Sauvageau, 2003; Park et al., 2003	Menichella et al., 1999; Murray et al., 1995; Pearce et al., 2004; Yamamoto et al., 1996; Wang et al., 2001; Kiel et al., 2005

*These are discussed later in the chapter Results and discussion (III and IV)

differentiated cells (Barker et al., 2007). The signalling regulating the choice between undifferentiation and differentiation within the progeny of a stem cell is not very well known. Several prominent mechanisms suggest that the fate of the progeny cells can be regulated by asymmetric compartmentalization of intracellular molecules during the division of a cell or the asymmetric division of a cell in the line of basement membrane (Wilson and Kotton, 2008). The epithelial stem cell niches in the crypt of the intestine and in the epidermis have been studied intensely, but also epithelial stem cell niches in continuously growing mouse incisor, hair and feather follicles have been under active research. The localisation of different epithelial stem cell niches are indicated in Figure 7. An overview of regulatory genes or signalling networks in several stem cell niches, including epidermis, feathers, hairs, intestine, bone marrow and mouse incisors is in Table 1.

Hematopoetic stem cells (HSCs) are among the most studied and best characterized stem cells. HSCs reside in the bone marrow of adults and can differentiate into all blood cell lineages (Orkin and Zon, 2008). The mature blood cells are short lived, thus the proliferation of the progenitors has to be effective, although HSCs are mainly quiescent.

5.3 Epidermal, hair follicle and feather stem cells

During adult homeostasis epidermis is mainly maintained by inter follicular epidermis (IFE) stem cells, residing in the basal layer (Ito et al., 2005). Stem cells responsible for hair follicle regeneration are located in a niche in the hair follicle bulge (Cotsarelis et al., 1990; Tumbar et al., 2004). However, after a wound hair follicle stem cells participate in the repairing of both epidermis and sebaceous glands (Morris et al., 2004; Levy et al., 2005; Levy et al., 2007). Single, cultured hair follicle stem cell can differentiate into all cell lineages of epidermis, thus they are able to reconstitute whole IFE (Blanplain

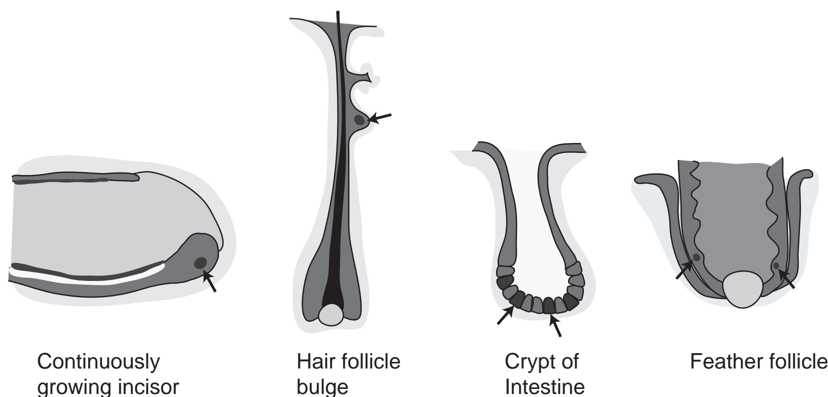

Figure 7.
Epithelial stem cell niches in a continuously growing incisor, in a crypt of intestine, in a hair follicle and in a feather follicle. The niches or stem cells are indicated by arrows. The epithelial stem cells of the incisor are located in the labial cervical loop. In the hair follicle stem cells are in the bulge and in the feather follicle they are in the collar bulge. The intestinal stem cells are the crypt basal cells.

et al., 2004; Rochat et al., 1994; Claudinot et al., 2005). In addition, progenitors of the isthmus and sebaceous glands are situated in the outer root sheath between the bulge and sebaceous gland (Ghazizadeh and Taichman, 2001; Nijhof et al., 2006).

Hair and feathers undergo continuous cycling maintained by stem cells, but they use different types of strategies to preserve their stem cells during moulting. Hair follicle stem cells migrate from the bulge to bulb during the growth stage and begin to proliferate (Cotsarelis et al., 1990; Tumbar et al., 2004). Stem cells give rise to all epithelial layers of the follicle during growth (Cotsarelis 2006; Fuchs, 2007). The activation and fate decision of the hair follicle stem cells are regulated by Wnts while BMP signalling functions as a regulator of quiescent (Table 1) (DasGupta and Fuchs, 1999; Blanpain et al., 2004; Kobielak et al., 2003, 2007; Andl et al., 2004).

Feathers do not have structure similar to the hair follicle bulge. Instead feather stem cells, identified as LRCs, are located in the collar bulge in the growing follicle and in the papilla ectoderm in the moulting follicle (Yue et al., 2005). Cell migration patterns between hair and feather are different and feather LRCs are not as quiescent as hair stem cells (Kopan et al., 2002; Morris et al., 2004; Yue et al., 2005). The more active proliferation in feather stem cells might be due to faster growth rate in the feather follicle compared to in the hair follicle. The localisation of LRCs also plays an essential role in the formation of radial symmetry in feathers (Yue et al., 2005).

5.4 Intestinal stem cells

Wnt, Shh and BMP signalling participate in the regulation of crypt development in the intestine. Recently several potential epithelial stem cell markers have been identified in the intestine, including Leucine rich repeat containing G-protein coupled receptor 5 (Lgr5), Prominin1 (also called CD133) and Bmi1 (Barker et al., 2007; Sangiorgi et al., 2008; Snippert et al., 2009). The common feature between these molecules is that they were already known as tumour associated genes.

Lgr5, an orphan G protein-coupled receptor, is a Wnt downstream target gene and was recently found to be a molecule specifically recognising the population of intestinal stem cells and the hair follicle stem cells (McDonald et al., 1998; Hermey et al, 1999; van der Flier et al., 2007; Barker et al., 2007; Jaks et al., 2008). The analyses of the crypt cell population in the intestine marked by Lgr5 showed that the cells had many characteristics of the stem cells including self-renewal and multipotency, but, surprisingly, the cells proliferated actively (Barker et al., 2007). However, the Lgr5 positive stem cells were crypt basal cells (CBCs) and not the +4 positioned cells earlier identified as stem cells. Recent results indicate that Lgr5 positive stem cells differentiate into four different cell types, and a single *Lgr5* expressing cell can generate all these cell types and form villus-like domains (Sato et al., 2009).

6. Stem cells in teeth

Albeit nearly all tissues have stem cells maintaining the normal tissue homeostasis, stem cells in the teeth, excluding continuously growing teeth, are only located in the mesenchyme, thus mesenchymal tissues are regenerated. The mesenchymal stem cells can differentiate for instance into odontoblasts, but the molecular mechanism regulating

differentiation remains unsolved despite many recently obtained advances. Epithelial stem cells are not maintained in most teeth and enamel forming ameloblasts are lost when the tooth erupts into the oral cavity. Although enamel is not replaced, the structure of the enamel as the hardest tissue of the mammalian body makes the tooth capable to endure plenty of exertion. None but continuously growing teeth maintain epithelial stem cells (Thesleff and Tummers, 2008).

Damage to or even loss of the teeth during a lifetime is common. The methods to repair the damage and replace the teeth artificially are nowadays well developed. However, even more natural and functionally superior alternatives are continuously under research, and new bioengineering methods have recently been developed. For instance, teeth can be cultured from dissociated and then recombined dental epithelial and mesenchymal cells *in vitro* and *in vivo*, and eventually they form a complete functional tooth (Nakao et al., 2007; Ikeda et al., 2009). Regeneration of a tooth can be manipulated with different stem cells together with dental structures *in vivo* (Duailibi et al., 2004). The regeneration of a certain tissue or even the whole tooth for clinical use has been the main goal in research concerning tooth stem cells. This goal can only be reached when further understanding of the stem cells has been uncovered.

Several different mesenchymal dental stem cells have been identified, including dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), dental follicle precursor cells (DFPCs) and apical papilla stem cells (SCAP) (Gronthos et al., 2000; Seo et al., 2004; Morsczeck et al., 2005a; Sonoyama et al., 2006). In addition, easily accessible stem cells from human exfoliated deciduous teeth (SHED) were isolated from pulp of human deciduous incisor and showed high plasticity in differentiation (Miura et al., 2003).

DPSCs were isolated from the pulp of the adult human teeth but their exact location and whether the stem cells are located in the exact niche or not remains unknown (Gronthos et al., 2000). The DPSCs have significant plasticity and are able to differentiate into odontoblasts as well as into several other mesenchyme derived pulp cells (Gronthos et al., 2002). Notch signalling has been suggested to have a role in mesenchymal stem cell regeneration (Mitsiadis et al., 1999). *In vitro*, BMP2 can induce differentiation of dental pulp stem cells into odontoblasts (Iohara et al., 2004).

Mesenchymal dental stem cells were also found from periodontal ligaments (PDL) (Seo et al., 2004). PDLSCs not only regenerate PDL, they also participate in the regeneration of alveolar bone and cementum. PDLSCs can differentiate for instance into cementoblast-like cells and adipocytes, and in transplantation experiments in rat molars PDLSCs formed a layer of cementum and collagen fibers (Seo et al., 2004).

DFPCs were isolated from the dental follicle and were able to differentiate into osteoblast, cementoblast, adipocyte and some even to neuronal like cell types (Morsczeck et al., 2005a; Morsczeck et al., 2005b; Ernst et al., 2009). DFPCs share some similar characteristics and behave similarly when induced to differentiate *in vivo* as PDLSCs (Morsczeck et al., 2005a; Morsczeck et al., 2005b). Recently, a population of mesenchymal stem cells from the root apical papilla (SCAP) were recognised in the human third molar (Sonoyama et al., 2006). SCAP

differentiate into odontoblast like cells which secrete dentin in transplantation experiments, as well as into adipocytes, and share some similar characteristics with DPSCs (Sonoyama et al., 2006). Evidence suggesting that SCAP are the source of odontoblasts responsible for formation of root dentin but SCAP might also play a role in dentin regeneration because they have better capacity for dentin regeneration than DPSCs (Sonoyama et al., 2006; Sonoyama et al., 2008).

6.1. Epithelial stem cell niche in the mouse incisors

The epithelial stem cell niche of a continuously growing mouse incisor is located in the stellate reticulum of the cervical loop (Harada et al., 1999). The labial cervical loop in a wt incisor is presumably enlarged due to the requirements for the active proliferation and differentiation of ameloblasts (Tummers and Thesleff, 2008). In the molar the stellate reticulum within the cervical loop is not maintained, the epithelial structures form HERS and the epithelial stem cells are lost. In the mouse incisor the stellate reticulum and the epithelial stem cells are maintained, and root formation does not occur. However, the two sides of the incisor are different, labial side covered with dentin and enamel, and lingual side with dentin and cementum. Nonetheless both labial and lingual cervical loop harbour stellate reticulum, albeit different sized, indicating that the HERS does not form (Tummers and Thesleff, 2008).

Epithelial stem cells in the incisors were identified within the labial cervical loop by LRC techniques, BrdU and DiI labelling. Five days after injection of fluorescent DiI to the centre of the labial cervical loop the cells containing the label had extended to the inner dental epithelium and were seen in the transient amplifying cells and differentiating ameloblasts (Harada et al., 1999). BrdU labelling indicated the label retaining cells in the cervical loop after seven days chase period (Harada et al., 1999). The localisation was confirmed with *Notch* expression in the slowly dividing cells in the cervical loop (Harada et al., 1999). However, although the epithelial stem cells were located in the cervical loop the exact site of the stem cell niche still remains unclear.

According to the current hypothesis, after the asymmetric proliferation of a stem cell one of the daughter cells will relaminate into the inner dental epithelium. In the inner dental epithelium the cell begins to proliferate actively and is hence named as a transient amplifying cell. After several proliferations the transient amplifying cell begins to differentiate first into a preameloblast and later into an ameloblast. Continuous production of ameloblasts in the proximal end and loss of matured ameloblasts in the apical end transfer the ameloblasts from proximal to apical direction, and the surrounding microenvironment regulates different functional stages of the ameloblast.

The epithelial stem cell niche in the cervical loop structurally resembles two other stem cell niches, the crypt of intestine and the hair follicle bulge (Moore and Lemischka, 2006). The crypt and the bulge, similar to the stellate reticulum in the cervical loop, form an epithelial compartment which is surrounded by mesenchymal tissue regulating the epithelium. Many stem cells can nowadays be recognised by specific marker genes but epithelial stem cells in the incisor still lack an identified marker gene.

6.2 Molecular regulation of epithelial stem cells in the incisors

The molecular regulation of continuously growing incisors resembles that of the molar, but expression of several signalling molecules, which are lost in the root forming molar, are maintained in the continuously growing incisor. These molecules are thought to participate in the maintenance of stellate reticulum and epithelial stem cells. FGF3, FGF10 and Notch1 have been suggested to be among these key molecules (Tummers and Thesleff, 2003). Notch signalling has been reported in association with stem cell regulation in many other tissues. In addition, the expression pattern of *Bmp4*, identical to *Fgf10* expression, suggests that it has a role in stem cell regulation (Tummers and Thesleff, 2003). *Fgf3*, *Fgf10* and *Notch1* expression are downregulated in the molar when crown development finishes and the root development begins, but their expression is maintained in the incisors and continuously growing sibling vole molar (Tummers and Thesleff, 2003).

In the incisor cervical loop Notch signalling pathway genes are expressed in a localised manner. *Notch1*, *Notch2* and Hairy and enhancer of split 1 (*Hes1*), the downstream target of Notch signalling, are expressed in the stellate reticulum and stratum intermedium, and *Jagged1* in the differentiating ameloblasts (Mustonen et al., 2002). In addition, *Notch2*, *Notch3* and *Notch4* are expressed in the epithelium (Mitsiadis et al., 1998). In the molar *Notch2* and Lunatic fringe (*Lfng*) signalling are maintained in P10 and P14 molars, but many other Notch pathway genes are downregulated in the cervical loop area (Tummers and Thesleff, 2003). Notch signalling is usually associated with keeping the cells quiescent, whereas FGFs stimulate proliferation. The role of Notch signalling in epithelial stem cell regulation in the incisor remains unknown but recent results suggest that inhibition of Notch signalling leads to decreased proliferation in the stellate reticulum and shrinkage of the cervical loop (S. Felszeghy, personal communication). However, this phenomenon can be reversed when the inhibition of Notch signalling is removed. Thus several results suggest that Notch signalling has an essential role in the stem cell regulation in the incisor.

Regulation of the epithelial stem cells can originate in the mesenchyme. *Fgf3* and *Fgf10*, expressed in the mesenchyme surrounding the cervical loop, induce the proliferation of the epithelium, and their receptor, *Fgfr2b*, is expressed in the epithelium of the cervical loop (Harada et al., 1999). FGF10 is suggested to be an essential regulator of epithelial cell as well as stem cell proliferation in the cervical loop because incisor development in *Fgf10*$^{-/-}$ mice is disrupted and the typical labial cervical loop structure is missing (Harada et al., 2002). *Fgf10*$^{-/-}$ mice die at birth due to absence of lungs, hence to analyse post natal incisor development the teeth were grown under a kidney capsule for three weeks (Sekine et al., 1999; Yokohama-Tamaki et al., 2006). The labial cervical loop in these incisors had lost the stellate reticulum and stem cells, and the HERS had formed (Yokohama-Tamaki et al., 2006). Thus, *Fgf10*$^{-/-}$ incisors grown under kidney capsule had lost the typical features of continuously growing incisors and resembled the molars, which have only limited growth period. In addition, when the level of FGF10 is increased in the molars the cervical loop structures are maintained (Yokohama-Tamaki et al., 2006). However, in *K14-Eda* incisor,

where the enamel is missing, both *Fgf3* and *Fgf10* expressions were present, although the labial cervical loop was diminished and the structure began to resemble root structures, suggesting that the root development can occur simultaneously as stem cells are maintained (Tummers and Thesleff, 2008). Analyses of *K14-Noggin* mice indicated that the size of the cervical loop of the incisor does not necessarily signify the capability of epithelial stem cells to differentiate into ameloblasts (Plikus et al., 2005). Inhibition of BMP signalling in *K14-Noggin* incisors caused enlarged cervical loops and over activated proliferation on both labial and lingual sides but the ameloblast differentiation was prevented. When another BMP inhibitor and modulator of Wnt signalling, Sostdc1, is lost, an extra incisor occurs (Murashima-Suginami et al., 2007; Munne et al., 2009).

In *Spry2*$^{+/-}$; *Spry4*$^{-/-}$ incisors lingual cervical loop is enlarged and ectopic ameloblast differentiation and enamel formation occurs on the lingual side at pn14 (Klein et al., 2008). A large lingual cervical loop is visible at E16.5 in *Spry2*$^{+/-}$; *Spry4*$^{-/-}$ and *Spry4*$^{-/-}$, and ectopic *Fgf* expression is detected close to the lingual cervical loop resembling the situation on the labial cervical loop (Klein et al., 2008). In the embryonic stage ameloblasts are detected in the incisor of both *Spry2*$^{+/-}$; *Spry4*$^{-/-}$ and *Spry4*$^{-/-}$, indicating that even minor unbalance of *Fgf* signalling causes lingual ameloblast differentiation. However, lingual ameloblasts are lost during the post natal development of *Spry4*$^{-/-}$, hence the lingual enamel is lost progressively (Boran et al., 2009). The ablation of *Spry4* is necessary for emergence of lingual ameloblasts, because downregulation of other *Spry* genes does not cause lingual ameloblasts (Klein et al., 2008). Tissue specific ablation of *Fgfr2* causes a diminished labial cervical loop and a lack of ameloblasts in the maxillary incisor, and also affects the odontoblast differentiation (Lin et al., 2009). Tbx1 has been associated with FGF signalling and deficiency in *Tbx1* causes a reduced labial cervical loop and ameloblast differentiation does not occur (Mitsiadis et al., 2008a; Catón et al., 2009). These results indicate the importance of correct balancing of FGF signalling during cervical loop formation and epithelial stem cell maintenance.

Recently, several other molecules were suggested to participate in the maintenance of stellate reticulum and epithelial stem cells. Deficiency of *Ctip2*, an enhancer of transcriptional repression, causes a diminished labial cervical loop in addition to defects in ameloblast differentiation (Golonzhka et al., 2009). Heparin-binding cytokines midkine (MK) and Heparin-binding growth-associated molecule (HB-GAM), which are associated with epithelial-mesenchymal interactions during development, are expressed close to or in the cervical loops during tooth development (Mitsiadis et al., 2008b). In addition, both MK and HB-GAM induce proliferation in the cervical loop area, and MK and HB-GAM interact with several molecules, for instance BMPs and FGFs.

AIMS OF THE STUDY

The aim of this thesis was to analyse the molecular mechanisms regulating tooth development and especially the regulation of epithelial stem niche in the incisor. The specific aims were:

1. To examine the role of TGFβ signalling during molar and incisor development using mice with loss of function and gain of function of *Fst*.
2. To study the function of TGFβ signalling and Fst in the regulation of epithelial stem cells in the continuously growing mouse incisor.
3. To localize Wnt pathway gene expression and Wnt/β-catenin activity in the epithelial stem cells of the incisor.

MATERIALS AND METHODS

Mouse strains

Strain	Used in article	Purpose
NMRI	I, II, III, IV	Analyse the expression patterns of indicated genes
Follistatin$^{-/-}$	I, II, III	Analyse the role of Follistatin deficiency in teeth development
K14-Follistatin	I, II, III	Analyse the role of Follistatin overexpression under Keratin14 promoter in teeth development
Fgf3$^{-/-}$	III	Analyse the teeth phenotypes of Fgf3 deficient mice
Fgf10$^{+/-}$	III	Analyse the teeth phenotypes of Fgf10 heterozygote mice
Fgf10$^{-/-}$	III	Analyse the teeth phenotype of mice deficient for both Fgf3 and Fgf10
K14-Noggin	III	Analyse the expression pattern of indicated gene
BATgal	IV	Active Wnt/β-catenin signaling analysis
TOPgal	IV	Active Wnt/β-catenin signaling analysis
Axin2$^{LacZ/LacZ}$	IV	Active Wnt/β-catenin signaling analysis

Methods used in articles I-IV

Method	Article
Histology	I, II, III, IV
Genotyping by PCR	I, II, III
Analysis of adult phenotype (skeletal preparation)	I, II, III
Radioactive *in situ* hybridization on sections	I, II, III, IV
Whole mount *in situ* hybridization	I, II, III
Tissue culture and bead experiments	I, II, III
Immunohistochemistry	I, II
Cell proliferation assay (BrdU)	I, III
LacZ staining	IV

Probes

The following probes were used for *in situ* hybridisation.

Probe	Reference	Used in article
ActivinβA	Erämaa et al., 1992	I, II, III
Alk3	Dewulf et al., 1995	III
Alk4	Verschueren et al., 1995	III
Ameloblastin	Lee et al., 1996	II
Axin2	Lammi et al., 2004	IV
Bmp2	Åberg et al., 1997	I, II, III
Bmp4	Åberg et al., 1997	I, II, III
Bmp7	Åberg et al., 1997	I, II, III
Dkk1	Andl et al., 2002	IV
Dkk2	Diep et al., 2004	IV
Dkk3	Fjeld et al., 2005	IV
Dkk4	Fliniaux et al., 2008	IV
dspp	D'Souza et al., 1997	II
Edar	Laurikkala et al., 2001	I
Fgf3	Kettunen et al., 2000	III
Fgf4	Jernvall et al., 1994	I
Fgf10	Kettunen et al., 2000	III
Follistatin	Wankell et al., 2001	I, II, III
Gdf11	Gift from Anne Calof (Univ. California)	I
Lef1	Kratochwil et al., 1996	IV
Lgr5	Barker et al., 2007	IV
Lrp4	Fliniaux et al., 2008	IV
mmp20	Gift from Dr. Jan Hu (Univ. of Texas)	II
p21	Jernvall et al., 1998	I, II
Shh	Vaahtokari et al., 1996	I
Tcf1	James et al., 2006	IV
Wnt2b	M. James	IV
Wnt3	Sarkar and Sharpe, 1999	IV
Wnt3a	M. James	IV
Wnt4	Sarkar and Sharpe, 1999	IV
Wnt5a	Sarkar and Sharpe, 1999	IV
Wnt5b	Sarkar and Sharpe, 1999	IV
Wnt6	Sarkar and Sharpe, 1999	IV
Wnt7a	M. James	IV
Wnt7b	Sarkar and Sharpe, 1999	IV
Wnt10a	Wang and Shackleford, 1996	IV
Wnt10b	Wang and Shackleford, 1996	IV
Wnt11	Sarkar and Sharpe, 1999	IV

RESULTS AND DISCUSSION

Follistatin has a role in the morphogenesis of the molars (I)

Follistatin (Fst) is an extracellular inhibitor of several TGFβ superfamily members, including BMPs and Activin (Nakamura et al., 1990; Iemura et al., 1998; Balemans and Van Hull, 2002). We studied the role of Fst during molar development by analysing two mouse models, loss of *Fst* function using *Fst*$^{-/-}$ mice and gain of *Fst* function using mice overexpressing Fst under a *Keratin14* promoter (*K14-Fst*) (Matzuk et al., 1995b; Wankell et al., 2001).

In situ hybridisation analyses of *Fst*, *Bmp2*, *Bmp4* and *Bmp7* expressions at E14 and E16 indicated overlapping expression patterns in the primary and secondary enamel knots. *Fst*, *Bmp4* and *ActivinβA* were also expressed in the papilla mesenchyme. BMPs participate in ameloblast (II; Coin et al., 1999) and odontoblast differentiation (Bègue-Kirn et al., 1992; Vainio et al., 1993). E18 differentiation stage expression of *Bmp2*, *Bmp4* and *Bmp7* was detected in the mesenchyme, most intensely in the preodontoblasts and odontoblasts, and weakly in the preameloblasts. The expression of *Fst* was restricted to the papilla mesenchyme in the vicinity of the follicle mesenchyme and cervical loops, while *ActivinβA* was expressed was exclusively in the follicle. Our results show that the expression patterns of *Fst*, *Bmp*s and *ActivinβA* were intense and partially overlapping during the molar development. The restricted expressions suggest important roles for Fst in the enamel knots and the development of tooth morphology.

The histological analyses of *Fst*$^{-/-}$ molars revealed that although the cap stage molar at E14 showed a normal tooth structure, in the early bell stage at E16 folding of the epithelium was already abnormal in both mandibular and maxillary molars. Because the primary enamel knot regulates the localisation of secondary enamel knots it is probable that the primary enamel knots were functioning already abnormally. The morphological differences between the wild type (wt) and *Fst*$^{-/-}$ molars were evident in crown morphology by E18. The cusps of the wt had become deep and had distinct anterior-posterior orientation, whereas the *Fst*$^{-/-}$ cusps were disoriented with shallow and aberrant cusp patterning, indicating abnormal folding of the inner dental epithelium. In addition, cell differentiation was affected. Differentiated odontoblasts were detected both in wt and *Fst*$^{-/-}$ molars at E18 but predentin formation had begun only in the wt. Ameloblasts had differentiated in the wt, but in the *Fst*$^{-/-}$ molars the cells of inner dental epithelium remained cuboidal and unpolarised. In addition, the usual, normally extensive extracellular tissue was missing from stellate reticulum, and instead the tissue was composed of closely packed cells. The analyses of inner dental epithelium proliferation by 5-bromo-2’-deoxyuridine (BrdU) labelling at E18 indicated that the amount of proliferating cells was diminished and the proliferating cells were irregularly distributed in the *Fst*$^{-/-}$ molar compared to the regular patterning of proliferation in the wt. To analyse the post natal phenotype of the *Fst*$^{-/-}$ molar we had to culture tissues *in vitro* because *Fst*$^{-/-}$ mice die shortly after birth (Matzuk et al., 1995b). Molars were dissected for

culturing at E17 and after 7 days culture, *Fst*$^{-/-}$ molars sustained the shallow and aberrant cusp pattern. However, ameloblast differentiation had proceeded, indicating that cell differentiation occurs, although delayed, even when *Fst* is lacking.

To further analyse the aberrant folding of the inner dental epithelium in *Fst*$^{-/-}$ molars we studied the secondary enamel knots. *p21*, a cyclin-dependent kinase inhibitor and enamel knot marker, was expressed in two secondary enamel knots in *Fst*$^{-/-}$ at E16. One of the enamel knots was enlarged, and folding of the inner dental epithelium was abnormal. The abnormal expression of *p21* and the affected cusp patterning in *Fst*$^{-/-}$ molar resembled that of *Sostdc1*$^{-/-}$ molar, with expanded expression in the enamel knot and altered cusp patterning (Kassai et al., 2005). In the primary enamel knot the analysed markers were expressed similarly both in the wt and *Fst*$^{-/-}$. However, the altered expression of secondary enamel knot marker as well as abnormal folding of the inner dental epithelium indicate that the function of the primary enamel knot was already altered resulting in defects in the formation and function of the secondary enamel knots. Both BMP4 (Jernvall et al., 1998) and ActivinA can induce *p21* expression in isolated dental epithelium *in vitro*. Our results suggest that Fst participates in the formation of secondary enamel knots by regulating the functions of BMPs and Activin.

In two month old *K14-Fst* mice cusp patterning was severely affected, showing aberrant cusp patterning or whirled occlusal surface in the most affected teeth, and worn enamel. The severity of the phenotype varied between different molars. The first molar showed less altered crown patterning than the second molar. In addition, the *K14-Fst* third molars were missing both from the upper and lower jaws. This may be caused by varying levels of *Fst* expression driven by *K14* promoter, or by differing sensitivity of the teeth to signalling regulated by Fst.

The primary enamel knot determines the localisation of the secondary enamel knots and thus participates in the formation of crown morphology (Jernvall and Thesleff, 2000). In the epithelium *Fst* was expressed in the area of both primary and secondary enamel knots, suggesting a role in enamel knot formation or functioning. At least in an *in vitro* situation both BMP4 (Jernvall et al., 1998) and ActivinA indeed induced enamel knot marker *p21* expression in isolated dental epithelium. p21, associated with cessation of proliferation and beginning of terminal differentiation, affects apoptosis of differentiated cells in the enamel knot (Jernvall et al., 1998). Activin regulates the expression of *Fst* in the dental epithelium and Edar in the enamel knot region (Ferguson et al., 1998; Laurikkala et al., 2002). Tooth development of *ActivinβA*$^{-/-}$ mice arrests at the bud stage, whith the exception of the upper molars, around the time the primary enamel knot is formed (Ferguson et al., 1998). This indicates that ActivinβA is essential for the transition from bud to cap stage. Although *Fst*$^{-/-}$ molar development did not arrest, our results show that Fst nontheless affects the primary enamel knot function because localisation of secondary enamel knots were abnormal, resulting in irregular and diminished proliferation and aberrant folding of inner dental epithelium.

Overexpression of *Fst* caused the loss of the third molar in both mandible and maxilla. The third molar is lost also at least in *Eda*$^{-/-}$ (Tabby) mutants, in mice producing low levels of *Pax9*, and in *K14-Noggin* mice (Pispa et al., 1999;

Kist et al., 2005; Plikus et al., 2005). In addition, overexpression of *Noggin*, an inhibitor of BMP signalling, caused abnormal ameloblast and odontoblast differentiation and altered crown pattern (Plikus et al., 2005). In addition, inhibition of *Bmp4* function by antisense oligodeoxynucleotide against *Bmp4 in vitro* caused abnormal cusp pattern and mildly affected ameloblast differentiation in the molar (Tabata et al., 2002). The absence of *Pax9* leads to downregulation of *Bmp4* in the mesenchyme at the bud stage and hypomorphic *Pax9* expression results in hypoplastic or missing lower incisors and third molars (Peters et al., 1998; Kits et al., 2005). The phenotype becomes increasingly severe with different forms of oligodontia as the level of *Pax9* expression is diminished. In addition to the loss of the third molar, the *Eda*$^{-/-}$ mutant mouse has abnormal cusp patterning resulting from downregulation of several essential genes expressed in the enamel knot, including Bmp4 (Grüneberg, 1971; Pispa et al., 1999). These results are consistent with ours (I, II) and confirm the important role of BMP signalling in the development of crown morphology. We suggest that the third molars in *K14-Fst* failed to form due to defects in dental tissue committed to the molar field, which then affected the last developing molar most severely. The same explanation was suggested for why the last teeth to develop are most often the missing teeth in humans (Nieminen et al., 2001, Nieminen, 2009). In contrast to the loss of molars, some mutations cause development of an extra molar, for example *K14-Eda*, *Spry2*$^{-/-}$ and *Spry4*$^{-/-}$ (Mustonen et al., 2003; Klein et al., 2005). The loss of *Sostdc1*, an inhibitor of BMP and modulator of Wnt signalling, induces the formation of both an extra molar and incisor (Kassai et al., 2005; Munne et al., 2009). Thus the unbalancing of BMP signalling can cause extra molars when its inhibition is absent, and a loss of molars when its inhibition is enhanced.

Our results show that Fst is an important regulator of cusp patterning and crown morphology, and that it has a role also in ameloblast differentiation. The complex phenotypes of *Fst*$^{-/-}$ and *K14-Fst* mutant mice indicate that negative regulation by Fst is essential in several different stages of tooth development. Both loss of *Fst* and overexpression of *Fst* cause aberrant crown morphology, albeit in different ways. Epithelial proliferation was downregulated, and differentiation and function of both odontoblasts and ameloblasts was delayed in *Fst*$^{-/-}$ mutants, indicating that the fine tuning of the signalling by inhibitor regulates different levels of tooth development. We suggest that *Activin*, expressed in the mesenchyme under the inner dental epithelium, induces *Fst* expression in the epithelium, and that Fst inhibits both Activin and BMPs, thus balancing and modulating enamel knot formation and crown morphogenesis. We have shown that Fst negatively regulates the differentiation of ameloblasts and that it modulates the morphogenesis of crown epithelium during molar development.

Follistatin regulates the asymmetric formation of enamel in the mouse incisors (II)

The morphology of a mouse incisor is distinctively different compared to the molar. Mouse incisors grow continuously throughout the animal's life to compensate for the wearing of the tooth, and the sharpness of the incisor tip is maintained by the asymmetric deposition of hard enamel. Ameloblasts differentiate and enamel is formed exclusively on the

labial side of the tooth, called the crown analogue, while the lingual side is covered by dentin and is called the root analogue.

Fst is an important regulator of cusp patterning and ameloblast differentiation in the molar, as we showed (I). The phenotype of the incisors in *K14-Fst* mouse at 1 month of age suggested that ameloblast differentiation and enamel formation are also altered in the incisor, although the phenotypes of the incisors were variable with different levels of severity. In the most severe cases the incisors were smaller and chalky white, with worn mandibular incisors and broken or overgrown maxillary incisors. The ground sections revealed loss of enamel from the entire incisor. The embryonic analyses of *K14-Fst* mice showed small and symmetric incisors. At E18 ameloblast differentiation had occurred on the labial side of the wt incisors, but the *K14-Fst* incisors lacked a polarised ameloblast layer. The odontoblast layer and dentin matrix was seen on both labial and lingual sides in wt and *K14-Fst* incisor. Because *Fst*$^{-/-}$ mice die perinatally (Matzuk et al., 1995b) the phenotypic characterisation of incisor was done at E18 stage. The ameloblast differentiation occurred on the labial side of *Fst*$^{-/-}$ incisor as in the wt, but in addition polarised ameloblasts were detected also on the lingual side together with enamel. The occurrence of lingual ameloblasts in *Fst*$^{-/-}$ incisors and the loss of ameloblasts in *K14-Fst* incisors were confirmed by analysing the expression patterns of several ameloblast marker genes. Although the lingual epithelial cells in *Fst*$^{-/-}$ incisor were confirmed as ameloblasts both histologically and functionally, the cell layer was not as well organised as the labial ameloblast layer. Since only partial rescue of lingual ameloblasts occurred in the *Fst*$^{-/-}$ mice, it is probable that another inhibitor of ameloblast differentiation exists on the lingual side, hence the partial compensation for the effects of Fst in *Fst*$^{-/-}$ incisor.

It has been shown previously that ameloblast differentiation can be induced in vitro by TGFβ1 and BMP2 (Coin et al., 1999), and that Fst is an inhibitor of BMPs and Activin (Nakamura et al., 1990). The distinct ameloblast differentiation phenotypes in *Fst*$^{-/-}$ and *K14-Fst* incisors suggest that Fst negatively regulates ameloblast differentiation. To confirm the role of Fst in ameloblast differentiation we analysed whether beads soaked in BMP2, BMP4, BMP7 or ActivinA protein can induce differentiation of ameloblasts in isolated incisor at E15 after 24hrs culturing *in vitro*. The differentiation of ameloblasts was verified by the expression of *p21*, which marks the cells differentiating into odontoblasts and ameloblasts (Bloch-Zupan et al., 1998), and by *ameloblastin*, an ameloblast marker gene in the epithelium. BMP2, BMP4 and BMP7 induced the expression of *p21* in the dental epithelium, but ActivinA had only weak stimulatory effect. In addition, BMP2, BMP4 and BMP7 induced epithelial expression of *ameloblastin*, whereas ActivinA induced only slight enhancement of endogenous expression of *ameloblastin*. Noggin, the specific inhibitor of BMP signalling, consistently caused downregulation of *ameloblastin* expression. The simultaneous exposure of the cultured incisors to Fst and BMP protein releasing beads caused downregulation of endogenous *ameloblastin*, and isolated *K14-Fst* incisor cultured with BMP4 bead showed reduced or loss of *ameloblastin* expression. Analyses of *Fst* induction confirmed that unlike BMP4, ActivinA induces *Fst* expression in E15 or E16 incisor after

24hrs of culturing. These experiments suggest that BMPs are the main inducers of ameloblast differentiation and that Fst is a negative regulator, thus inhibiting ameloblast differentiation on the lingual side.

To further investigate the roles of Fst, Activin and BMPs in ameloblast differentiation we analysed their expression patterns at E16 and E18. *Fst* was expressed at E16 in the epithelium on both labial and lingual sides. However, the expression was slightly asymmetric and became even more asymmetric at E18, when lingual side dental epithelium intensely expressed *Fst*, but in the labial side the expression was restricted to the outer dental epithelium and in the ameloblasts in the transitional stage. *Fst* expression was also detected in the P4 mouse molars in the tips of the cusps where ameloblast differentiation does not occur. *ActivinβA* and *Bmp4* expressions were restricted to the mesenchyme, and were especially intense in the papilla mesenchyme lining the cervical loop at E16. *Bmp*s were also intensely expressed in the odontoblasts. Comparisons of the expression patterns of *Fst*, *ActivinβA*, *Bmp2*, *Bmp4* and *Bmp7* in the incisor suggested close interaction between Fst and ActivinβA or BMPs, and, consistent with our earlier results, indicated that Fst is a negative regulator of ameloblast differentiation.

The continuously growing mouse incisor is an interesting model in which to study ameloblast proliferation and differentiation because ameloblasts at different developmental stages can be followed from proximal to distal area of incisor. Our results in mouse incisors suggest that Fst inhibits ameloblast differentiation in the lingual side. When the inhibition is lost in *Fst*$^{-/-}$, the asymmetry of ameloblast differentiation and enamel deposition is lost and enamel is formed on both labial and lingual side. Consistent with this is the loss of ameloblast and enamel on the labial side of the incisor in *K14-Fst*. Thus Fst negatively regulates ameloblast differentiation and is responsible for the asymmetric enamel deposition only on the labial side of the incisor. The regulation of ameloblast differentiation occurs through the inhibition of BMP function, but not through Activin, because the targets of negative regulation by Fst during ameloblast differentiation are BMPs. The variable phenotype in *K14-Fst* incisors suggests that a high level of Fst is needed to achieve the severe phenotype. Fst binds Activin with higher affinity than BMP, suggesting that the inhibition of BMPs requires higher *Fst* expression than Activin inhibition. Although ameloblasts differentiated and formed enamel on the lingual side of *Fst*$^{-/-}$ incisor, the ameloblast layer was disorganised, suggesting that another inhibitor partially compensates Fst function on the lingual side. Our results suggest that the dental follicle, in addition to dental epithelium and dental papilla, plays a role in ameloblast differentiation. Activin, expressed in the dental follicle, induces Fst in the dental epithelium. This indicates that dental follicle has a role in regulation of dental epithelium, which has not been demonstrated earlier.

Tissue recombination experiments have shown that the lingual dental epithelium looses the competence to differentiate into ameloblasts (Amar et al., 1989), and we showed that Fst is responsible for this. If Fst expression is lost, as in *Fst*$^{-/-}$ incisor, lingual epithelium is also competent to differentiate into ameloblasts. In humans, a heterogenous group of inherited defects in the enamel called Amelogenesis imperfecta (AI) can be caused for example by changes in

ameloblast specific genes (Gibson et al., 2001; Fukumoto et al., 2004). In addition, there are examples of animals lacking dental enamel, such as the sloth, and several known mutations in mice cause the absence of enamel in incisors, such as *K14-Eda*, *K14-Edar*, *K14-Noggin*, *K14-Wnt3* and *Tbx1*$^{-/-}$ (Mustonen et al., 2003; Pispa et al., 2004; Tucker et al., 2004; Plikus 2005; Millar et al., 2003; Mitsiadis et al., 2009; Tummers and Thesleff, 2008). In *K14-Noggin* incisors the BMP pathway is inhibited, thus resembling the situation in *K14-Fst* mice. However, *K14-Fst* incisors are slightly smaller than wt, while *K14-Noggin* incisors are enlarged (Plikus et al., 2005). Despite the size difference, both mouse mutants lack incisor enamel, supporting the role of BMPs in inducing ameloblast differentiation. In *K14-Eda* incisors the enamel is lost but in Tabby mice the enamel is accumulated on wider area (Tummers and Thesleff, 2008; Risnes et al., 2005). In addition, Eda can inhibit BMP4 function by stimulating the expression of *Fst* together with another BMP antagonist, Ccn2 (CTGF) in the incisor (Pummila et al., 2007). On the other hand, unbalancing FGF signalling in *Spry4*$^{-/-}$ and *Spry4*$^{-/-}$;*Spry2*$^{+/-}$ incisors causes ectopic ameloblast differentiation and enamel deposition on the lingual side (Klein et al., 2008). Thus BMP signalling, and especially BMP4, is a key inducer of ameloblast differentiation, through many molecules, at least Eda, Wnts and FGFs, participate in fine tuning of the regulation.

Based on our results, we have identified a regulatory network directing the formation of asymmetric ameloblast differentiation and enamel formation in the incisor. Three dental tissues participate in the interaction; the follicle mesenchyme, the dental epithelium and the papilla mesenchyme. The mesenchymal tissues have opposite effects on the epithelium, where the regulation of ameloblast differentiation occurs. Activin from the

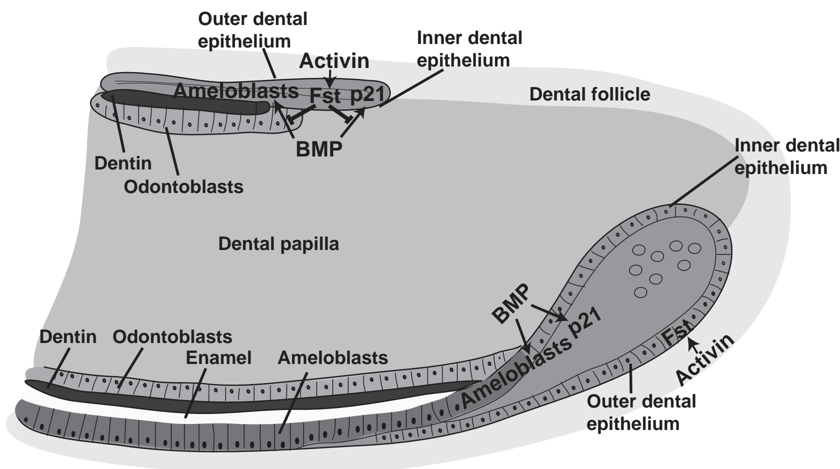

Figure 8.
Asymmetric expression of *Fst* regulates ameloblast differentiation in the incisor. On the labial side BMP from the dental papilla induces expression of p21and differentiation of ameloblasts. However, on the lingual side Activin from the dental follicle induces *Fst* expression in the epithelium and Fst inhibits BMP. Thus, BMP cannot induce ameloblast differentiation.

dental follicle induces *Fst* expression in the epithelium, and Fst inhibits the inductive signalling of BMP derived from the papilla to the epithelium (Figure 8). Thus Activin inhibits BMPs to induce ameloblast differentiation indirectly through *Fst* induction. This occurs on the lingual side, and results in lack of ameloblast differentiation and enamel formation. On the labial side, *Activin* expression becomes restricted mainly to the dental follicle at E18, leading to Fst's limited expression in the outer dental epithelium, and its inability to inhibit BMPs in the dental papilla. This enables BMPs to induce ameloblast differentiation, and the formation of asymmetric enamel deposition in the incisor.

A signalling network consisting of BMPs, Activin, FGFs and Follistatin regulates the epithelial stem cell niche in the incisor (III)

The continuous growth in the mouse incisors is maintained by stem cells. The epithelial stem cells in the incisor have been shown to reside in the proximal part of the incisor, in the cervical loop (Harada et al., 1999). The labial cervical loop maintains actively proliferating cells which differentiate into ameloblasts within a large stellate reticulum, whereas lingual cervical loop is small with less proliferation and fewer cells. During our analyses of ameloblast differentiation in *K14-Fst* incisor we noticed that in addition to the small size and loss of ameloblasts from the labial side, the incisors had lost the labial lingual asymmetry of the size of the cervical loops. *K14-Fst* incisor resembled the phenotype seen in *Fgf10*$^{-/-}$ incisors, and in addition FGF10 signalling has been closely associated with epithelial stem cell regulation (Harada et al., 1999; Harada et al., 2002).

Histological analysis of proximal area of *K14-Fst* incisor at E18 showed that in addition to small size and lack of enamel, the typical large stellate reticulum compartment in the labial cervical loop was absent and the cervical loop was diminished. Surprisingly, in the *Fst*$^{-/-}$ incisor the lingual cervical loop was enlarged and contained stellate reticulum and differentiating ectopic ameloblasts, thus resembling of labial cervical loop in wt incisor. Proliferation analyses with BrdU indicated that in the wt incisor the proliferating cells were most abundant in the inner dental epithelium on the labial side cervical loop, whereas on the lingual side proliferation was sparse. However, in *K14-Fst* the proliferation was decreased in the labial cervical loop and in *Fst*$^{-/-}$ proliferation on both lingual and labial cervical loops was increased. These results suggest that Fst has an important role in the proliferation of dental epithelial cells in the cervical loops and in regulating the formation of an asymmetric structure.

FGF signalling has been connected with the regulation of epithelial stem cells (Harada et al., 1999; Harada et al., 2002). Mainly FGF10 has been suggested to regulate epithelial proliferation, but expression pattern analyses have shown that *Fgf10* is expressed symmetrically in the papilla at E18 aligning both the labial and lingual cervical loops (Harada et al., 1999). However, *Fgf3* expression is asymmetric and the expression is restricted to the papilla next to the labial cervical loop, partially overlapping with *Fgf10* at E18. We analysed the expression patterns of *Fgf3* and *Fgf10* in *K14-Fst* and *Fst*$^{-/-}$ incisors. In *K14-Fst* incisor *Fgf3* expression was downregulated. Conversely in *Fst*$^{-/-}$ incisors ectopic

expression was detected in the papilla close to the enlarged lingual side cervical loop. *Fgf10* expression was similar in the mutants and wt. Proliferation analyses *in vitro* confirmed that in addition to FGF10, FGF3 can induce proliferation in the dental epithelium. According to these results Fst inhibits the expression of lingual *Fgf3* in the wt and indirectly affects the proliferation of the dental epithelium.

FGF signalling is an essential regulator of epithelial proliferation in the incisor. Downregulation of *Fgf* signalling in *Fgf10*$^{+/-}$ mouse teeth does not cause a phenotype differing from the wt, but in *Fgf10*$^{-/-}$ incisor the labial cervical loop is hypoplastic (Harada et al., 2002). We analysed *Fgf3*$^{-/-}$ and *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ compound mutant incisors and detected differentiated ameloblasts at P1. However, the labial cervical loop in *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ incisor was slightly smaller than in wt and *Fgf3*$^{-/-}$. At 5 weeks of age both *Fgf3*$^{-/-}$ and *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ incisors had distinct phenotype differing from the wt. Incisors in both mutants were white, but *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ incisors were also smaller and broken in several mice suggesting defects in the enamel. Ground sections of the incisor confirmed that in *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ enamel was missing, and although enamel was seen in *Fgf3*$^{-/-}$ incisor, the white colour of the incisor and the worn cusps of the molars suggested defects in the enamel. FGF3 and FGF10 seem to function cooperatively during tooth development, because the greater the number of alleles lost, the more severe the enamel phenotype. Consistent with this is the molar phenotype of *Fgf3*$^{-/-}$*; Fgf10*$^{-/-}$, in which development arrests at the bud stage.

Ectopic expression of *Fgf3* was seen in the *Fst*$^{-/-}$ incisor, suggesting interaction of pathways at some level. To further study these interactions we analysed the expression of *Fgf3*, *Fst*, *ActivinβA*, *Bmp2*, *Bmp4* and *Bmp7* in the developing incisor from E14 to E16. *Fgf3* expression became asymmetric in the papilla mesenchyme close to the labial cervical loop at E16. *ActivinβA* expression was already asymmetric at E15, when expression also became intense also next to the labial cervical loop. Thus *Fgf3* and *ActivinβA* had overlapping expression patterns at E15. *Bmp4* was expressed in the mesenchyme symmetrically around both labial and lingual cervical loops, and in the preodontoblasts. *Fst* was extensively expressed in the lingual dental epithelium, but in the labial epithelium expression became restricted to the outer dental epithelium at E16. The expression pattern of *Fst* suggests that the inhibitory effect of *Fst* to the papilla is more prominent on the lingual side than on the labial side due to distance from the papilla. Activin and BMP receptors *Alk3* and *Alk4* were expressed in the epithelium, indicating that BMP and Activin signalling regulate the epithelium.

Fst functions as an inhibitor of Activin and BMPs, and we showed that BMP4 induces ameloblast differentiation and Fst negatively regulates its induction (II). In addition upregulation or downregulation of *Fst* altered FGF signalling. Because of these results we were interested in analysing the role of BMPs and Activin in FGF signalling in the incisor. Surprisingly beads soaked in BMP4 inhibited *Fgf3* expression in incisors isolated at E16 and cultured for 24hrs. However, BMP4 had no effect on *Fgf10* expression. Noggin caused ectopic expression of *Fgf3* in the papilla mesenchyme on the lingual side next to the epithelium. A similar pattern of *Fgf3* expression was also detected in *K14-Noggin* mice, as well as in *Fst*$^{-/-}$ incisors.

These results confirm that BMP4 inhibits *Fgf3* expression. Thus both Fst and BMP4 inhibit the expression of *Fgf3*, functioning synergistically unlike in ameloblast differentiation, where Fst and BMP4 have antagonistic effects (II). The search for antagonistic signal for BMP4, hence inducing *Fgf3* expression on the labial side in wt incisor and regulated by Fst lead us to study the effects of Activin on *Fgf3* expression. *In vitro* cultures revealed that similar to Noggin, ActivinA was also able to induce ectopic *Fgf3* expression on the lingual side papilla mesenchyme next to the dental epithelium, albeit not so strongly as Noggin. Cell proliferation analyses by BrdU confirmed that ActivinA induces the proliferation of dental epithelium in the isolated incisor, and when endogenous Activin/TGFβ signalling was prevented by selective inhibitor of ALK receptors (SB431542) the labial cervical loop was diminished containing few epithelial cells. However, the effects became significant only after 4d of culture. These results indicate that Activin and BMPs antagonistically regulate *Fgf3* expression in the mesenchyme and the proliferation of epithelial cells, including stem cells, in the cervical loops.

To analyse the mechanisms regulating the expression of *Activin* and *Fgf3* in the dental mesenchyme we cultured the incisors at E14 or E15 for 24hrs. Neither *Fgf3* nor *Activin* was expressed in the separated dental mesenchyme, but placing dental epithelium beside the separated mesenchyme induced mesenchymal expression of both *Fgf3* and *Activin*. However, the expression of both genes was restricted in the mesenchyme aligning the epithelium. Direct regulation between Activin and FGF3 in the isolated mesenchyme did not occur, thus Activin was not able to induce *Fgf3* or *vice versa* in the dental mesenchyme, indicating that the regulation occurs through the dental epithelium. However, FGF9, expressed in the dental epithelium, was able to induce the expression of both *Activin* and *Fgf3*. Nonetheless, it is unlikely that FGF9 could be exclusively responsible for the restricted expression patterns of both *Activin* and *Fgf3* beginning from the E15 and E16, because *Fgf9* becomes asymmetrically expressed only after E18 (Kettunen and Thesleff, 1998).

We showed earlier that Fst negatively regulates ameloblast differentiation by inhibiting BMP signalling on the lingual side of the incisor, thus causing the labial-lingual asymmetry in enamel formation (II). The interaction occurred between follicle mesenchyme, dental epithelium and papilla mesenchyme, indicating active epithelial mesenchymal interaction. Our results concerning the regulation of epithelial stem cells in the cervical loops also showed interaction between these tissues, emphasizing the essential role of epithelial mesenchymal interaction. Epithelial stem cells in the incisors have been located in the cervical loop but the exact stem cell population remains unclear (Harada et al., 1999). Several genes have been suggested to participate in the regulation of incisor stem cells, such as FGF10, FGF3, BMP4 and Notch1 (Harada et al., 1999, 2002; Tummers and Thesleff, 2003).

According to our results FGF3 together with FGF10 maintain the epithelial stem cell pool in the labial cervical loop. Asymmetric *Fgf3* expression is not necessary for the formation of cervical loop but it seem to cause the epithelial proliferation and larger stellate reticulum in the labial cervical loop than in the lingual side. This can be confirmed by the ability of FGF3 to induce proliferation in

the dental epithelium, and the asymmetric expression pattern of *Fgf3*, consistent with the labial lingual asymmetric structure. Besides, when *Fst* is downregulated in the *Fst*$^{-/-}$ incisor, ectopic *Fgf3* expression is detected lingually next to the enlarged cervical loop. Our results suggest that whereas moderate proliferation of lingual cervical loop is maintained by FGF10 signalling, the active proliferation and maintenance of stem cells of labial cervical loop is regulated by cooperative function of both FGF10 and FGF3. Thus the role of FGF10 might be in the maintenance of basal level of stem cells essential for continuous growth. In *Fgf10*$^{-/-}$ the labial cervical loop is diminished and stellate reticulum is missing, but ameloblast differentiation had occurred at E19 (Harada et al., 2002). However, after three weeks of growth under the kidney capsule the ameloblast layer was lost, most likely due to loss of the stellate reticulum and stem cells leading to absence of ameloblast layer maintenance (Yokohama-Tamaki et al., 2006). The defects in the cervical loop structure in *K14-Fst* are more severe than in *Fgf3*$^{-/-}$, suggesting that Fst affects other molecules in addition to FGF3. Also, in *Tbx1*$^{-/-}$ mouse incisor the ameloblasts are not differentiated and the labial cervical loop is small missing the stellate reticulum resulting from the down regulated proliferation (Catón et al., 2009). FGF can induce *Tbx1* expression in the preameloblasts and in *Spry2*$^{+/-}$*;Spry4*$^{-/-}$ incisors *Tbx1* expression is detected not only on the labial side epithelium as in wt but ectopically on the lingual side epithelium as well, thus linking FGF and Tbx1 signalling (Mitsiadis et al., 2008; Catón et al., 2009). When Ctip2, an enhancer of transcriptional repression, is deficient in the incisor, the labial side ameloblast layer is defective and the stellate reticulum is diminished (Golonzhka et al., 2009). Surprisingly, ameloblast differentiation also occurs on the lingual side and epithelial expansion was detected. The mice die soon after birth and the incisor phenotype after eruption remains unclear (Wakabayashi et al., 2003). Table 2 presents the genes which have been suggested to have a role in epithelial stem cell regulation or have been shown to cause a phenotype in the incisor. The genes are divided according to whether they were known before or have been found after the article III was published and also our results from the article III are mentioned.

Our results suggest that FGF, BMP, Activin and Fst are integrated in the signalling network balancing the proliferation of dental epithelial stem cells and transient amplifying cells in the stellate reticulum of labial cervical loop. Mesenchymal FGF3 and FGF10 are the inducers of epithelial cell proliferation. BMP4, expressed in the mesenchyme next to both the labial and lingual cervical loops, did not have any effect on *Fgf10* expression, but *Fgf3* expression was repressed. This suggests that FGF signalling regulated by BMP and Activin exists only through FGF3. The first sign of a signalling network is seen at E15, when *ActivinβA* becomes expressed asymmetrically in the mesenchyme, intensive expression detected close to the labial cervical loop. Activin might antagonise the effects of BMP4 and thus causing the labial expression of *Fgf3* to remain after E16, when *Fgf3* expression is downregulated elsewhere in the papilla mesenchyme. This indicates that two TGFβ signalling family members antagonistically regulate epithelial stem cell proliferation through the regulation of *Fgf3* expression. Fst negatively regulates

Table 2.
Potential incisor epithelial stem cell fate regulators. The upper part of the table shows the earlier found molecules and their effects as well as the results presented in the article III. The lower part of the table shows the molecules which have been recognised after the article III was published.

Participating signals	Role	References	Results in article III
Notch1	Regulation of stem cell proliferation	Harada et al., 1999; Tummers and Thesleff, 2003	
Notch2	Regulation of stem cell proliferation	Harada et al., 1999	
FGF3	Expression patterns suggest a role in epihtelial stem cell regulation	Tummers and Thesleff, 2003	Induces the proliferation of epihtelial stem cells
FGF10	Induces proliferation in the cervical loop	Harada et al., 1999; Tummers and Thesleff, 2003	
BMP4	Expression patterns suggest a role in epihtelial stem cell regulation	Tummers and Thesleff, 2003	Inhibits epithelial stem cell proliferation through antagonizing the expression of *Fgf3*
Activin			Antagonizes the inhibitory effect BMP4 has on *Fgf3* expression
Fst			Regulates proliferation of epithelial stem cells by antagonising Activin signalling

Participating signals	Role	References
Spry2, Spry4	Downregulation of FGF inhibition causes an enlarged lingual cervical loop	Klein et al., 2008
Tbx1	Loss of Tbx1 causes a reduced labial cervical loop	Catón et al., 2009
Ctip2	Loss of Ctip2 causes a reduced labial cervical loop	Golonzhka et al., 2009
MK	Induces proliferation in the cervical loop area	Mitsiadis et al., 2008b
HB-GAM	Induces proliferation in the cervical loop area	Mitsiadis et al., 2008b

Activin on the lingual side, thus enabling BMP4 to inhibit *Fgf3* expression in the mesenchyme. However, on the labial side *Fst* expression is restricted in the outer dental epithelium and the distance to papilla, where *Activin* is expressed, confines the negative regulation by Fst. This allows Activin to counteract BMP4 and thus enable strong *Fgf3* expression. This signalling network explains the formation of labial lingual asymmetry in the cervical loops and the increased number of labial epithelial stem cells.

Several experiments indicated that the regulation of *Fgf3* by Activin and BMP is indirect and functions through the epithelium. Tissue culture experiments showed that *Fgf3* was lost in the separated mesenchyme, and that expression was maintained if mesenchyme and epithelium were recombined. ActivinA was not able to rescue *Fgf3* expression in the separated mesenchyme and was only able to induce epithelial proliferation after 4 d of culture. In addition, the expression pattern of *Fgf3* in the cultured incisor, detected in the mesenchyme underlying lingual epithelium and not that surrounding the bead, suggests that the epithelium is essential and that signalling proceeds through epithelium. Convergent with this is the analysed expression patterns of Activin and BMP receptors, which were detected only in the epithelium. These results show that Activin induces *Fgf3* expression but the regulation functions through the epithelium (Figure 9). BMP4 inhibited the expression of *Fgf3* in the cultured incisor, but the expression was rescued by ActivinA. In addition to ActivinA inducing ectopic expression close the lingual dental epithelium, inhibition of BMP by Noggin was able to mimic this phenomenon.

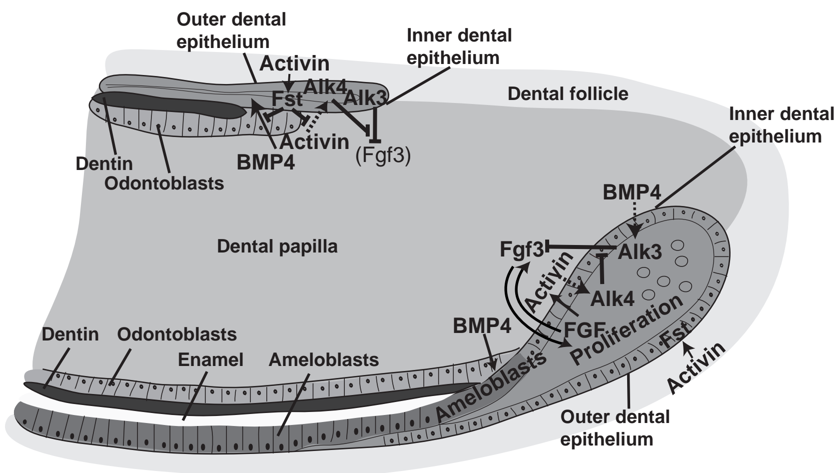

Figure 9.
A signalling network regulating epithelial stem cells in the mouse incisor. *Fgf3*, which together with FGF10 regulate epithelial stem cell proliferation, is expressed in the dental papilla on the labial side. BMP4 inhibits *Fgf3* expression, but Activin antagonises this effect, thus *Fgf3* is expressed and epithelial stem cell proliferation is maintained on the labial side. However, on the lingual side Fst negatively regulates Activin and BMP4 is able to inhibit *Fgf3* expression, therefore the amount of stem cells is limited.

Consistent with this is the phenotype of *K14-Noggin* incisor where proliferation is increased in both labial and lingual dental epithelia (Plikus et al., 2005). Schematic pictures of different incisor phenotypes of several mouse mutants are in Figure 10. Our results suggest that BMP4 inhibits *Fgf3* expression, but that Activin can counteract the inhibition and hence *Fgf3* is expressed. This occurs on the labial side, while on the lingual side Fst inhibits Activin, which allows BMP negatively regulate *Fgf3* expression and the epithelial proliferation remains low in the lingual cervical loop. Both BMP and Activin regulate *Fgf3* expression indirectly through the epithelium. The signalling that induces the expression of *Fgf3* and *Activin* remains unsolved. One candidate, FGF9, is expressed particularly in the epithelium in the cervical loop, and it is able to induce both *Activin* and *Fgf3* expression in the separated mesenchyme. However, some other signal is needed in addition to FGF9 because the asymmetric expression of *Activin* and *Fgf3* is formed before *Fgf9* expression becomes asymmetric and restricted only in the labial cervical loop. It is possible that another FGF could be a participant, because FGFs are functionally redundant.

Expression of Wnt pathway genes and Wnt/β-catenin activity in the incisors indicate absence of Wnt/β-catenin signalling in the epithelial stem cells (IV)

The Wnt signalling family, an essential family known to participate in tooth development, has not been analysed much during the incisor development, although it is known to have important

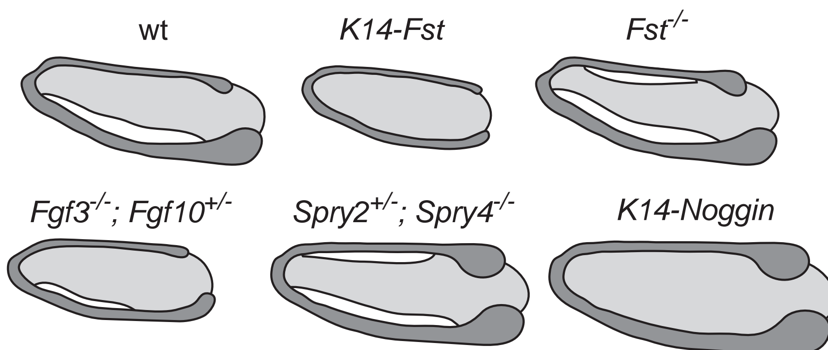

Figure 10.
Several mouse mutants display different phenotypes in the size of stem cell niches of the cervical loops. A wild type incisor presents a typical asymmetry with a large labial cervical loop as well as labial distribution of enamel. In *K14-Fst* incisor this asymmetry is lost, thus labial and lingual cervical loops are equally sized and enamel is absent. Asymmetry is absent also in *Fst*$^{-/-}$ incisor, where lingual cervical loop is enlarged and enamel is formed on both labial and lingual side. In *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ incisor labial cervical loop is hypoplastic but is able to maintain ameloblasts and enamel formation for a short period. When FGF signalling is increased in *Spry2*$^{+/-}$*; Spry4*$^{-/-}$ incisor differences between labial and lingual cervical loops decrease and enamel is formed on both sides. When BMP signalling is inhibited in *K14-Noggin* incisor the asymmetry is lost; both labial and lingual cervical loops are enlarged and enamel is missing. Dental epithelium in dark grey, enamel in white.

roles in stem cell maintenance or in the proliferation and differentiation of stem cells, depending on the context. We analysed the expression patterns of 12 Wnt ligands as well as several inhibitors, signal mediators and targets, in the incisor at E16 and E18. The analysed Wnt ligands were *Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt6*, *Wnt7a*, *Wnt7b*, *Wnt10a*, *Wnt10b* and *Wnt11*, of which *Wnt2b* and *Wnt3* were not detected in the incisor. Most Wnt ligands were detected in the dental epithelium at E16 and /or E18, and some were strongly expressed in the differentiating odontoblasts and ameloblasts. The expression patterns varied but unexpectedly none of the Wnts were expressed in the labial cervical loop, where putative epithelial stem cells reside. However, *Wnt4* and *Wnt6* were detected in the lingual cervical loop and *Wnt5a* was intensely expressed in the mesenchyme aligning lingual cervical loop but only weakly around the labial cervical loop. *Wnt3a*, *Wnt4*, *Wnt6* and *Wnt10a* were expressed in the preameloblasts and ameloblasts. At E18 *Wnt6* and *Wnt10a* were intensely expressed in the odontoblasts.

The analysed Wnt pathway mediators and inhibitors included transcription factors Lef1 and Tcf1, three antagonists of the Wnt/β-catenin pathway Dkk1, Dkk2 and Dkk4, and two as of yet functionally controversial genes Lrp4 and Dkk3 (Behrens et al., 1998; Ohazama et al., 2008; Caricasole et al. 2003; Hoang et al. 2004; Niehrs, 2006; Lee et al. 2009). *Dkk4* expression was not detected in the incisor, although it was present in the molar (Fliniaux et al., 2008). Also Dkk1, Dkk2 and Dkk3 are expressed in the molar (Fjeld et al., 2005). None of the analysed Wnt mediators or inhibitors was detected in the epithelium of the labial cervical loop. The expressions were mainly seen in the mesenchyme, except *Lrp4* and *Dkk3* expression, which were seen in the dental epithelium. *Lef1* and *Tcf1* were expressed in the mesenchyme close to the labial and lingual cervical loops, and *Tcf1*, *Lrp5* and *Dkk1* were expressed intensely in the odontoblasts. *Axin2*, a negative regulator and downstream target of Wnt/β-catenin signalling, was expressed in the lingual epithelium and weak expression was also seen in the preameloblasts and ameloblasts in the labial side. In the mesenchyme *Axin2* expression was intense, detected close to the labial and lingual cervical loops and in addition to in the preodontoblasts and odontoblasts at E16 and E18. To further study Wnt signalling in developing incisor we analysed active Wnt/β-catenin signalling with three different Wnt reporter mouse lines, *BATgal*, *TOPgal* and *Conductin/Axin2*$^{lacZ/lacZ}$ (DasGupta and Fuchs, 1999; Lustig et al, 2002; Maretto et al., 2003). *BATgal* and *TOPgal* lines have several mulitimerised TFC/Lef binding sites and siamois or c-fos minimal promoter driving *LacZ* expression (DasGupta et al., 1999; Maretto et al., 2003). The reporter lines indicate active Wnt signalling by expressing the enzyme β-galactosidase from *LacZ* gene in cells where the transgene is activated. *Axin2* expression and *BATgal* and *TOPgal* reporter activity were all missing from the labial cervical loop. In addition, reporter activity was missing from the entire dental epithelium, thus the reporter activity was slightly different although partially overlapping with the *Axin2* expression and some variability was seen also between the reporter lines. *BATgal* reporter was detected in the papilla, but most intensely in the preodontoblasts and odontoblasts, and also in the cells surrounding cervical loops. *TOPgal* was also active in the

odontoblasts, and in the cells underlying odontoblast layer. The expression of *Axin2* was intense and distributed more widely than the reporter line activities in the mesenchyme. *Axin2* expression was also seen in the dental epithelium. These observations indicate that reporter activity was variable but partially overlapping, and *Axin2* expression was seen even in the wider areas. Similar observations have been made in other tissues as well, suggesting that the reporter lines might show different activity depending on the transgene and fail to locate low levels of Wnt/β-catenin signalling (Boras-Granic and Wysolmerski, 2008; Barolo, 2006). Wnt signalling might participate in preodontoblast and odontoblast differentiation and functioning, but it does not seem to have a role in the maintenance of epithelial stem cells in the incisor.

Lgr5, a Wnt target gene associated with intestinal and hair stem cells (Barker et al., 2007; Jaks et al., 2008), was expressed in the incisor both at E16 and E18. The expression pattern was localised in the epithelium of the cervical loop, in a small compartment of cells within the stellate reticulum of labial cervical loop. The area corresponds to the site where putative epithelial stem cells are thought to reside. This was the only site of expression in the incisor at these stages. Because no Wnt activity was seen in the *Lgr5* expressing cells it is possible that in the incisor Lgr5 is not a direct Wnt target gene.

Wnt signalling has an essential role the tooth development. Alterations in Wnt signalling affect tooth development already in the early stages. Inhibition of Wnt signalling, as in *Lef-/-* mice, arrests tooth development at the bud stage because mesenchymal β-catenin is essential for transition from bud to cap stage (van Genderen et al., 1994; Chen et al., 2009). On the other hand, continuous activation of Wnt/β-catenin signalling in the oral epithelium induces ectopic teeth and continuous tooth formation (Järvinen et al., 2006; Liu et al., 2008). Our results show that analysed Wnt ligands, inhibitors or mediators are expressed extensively in the incisor, but nonetheless all of them are absent from the labial cervical loop where epithelial stem cells reside. In addition, active Wnt/β-catenin signalling was missing from the epithelium. However, *Lgr5*, an epithelial stem cell marker in the intestine and hair follicle was expressed in the labial cervical loop, suggesting this as the location of the epithelial stem cell niche.

We detected the expression of 10 Wnt ligands in the developing incisor at the cell differentiation stage at E16 and E18, possibly participating in the cellular differentiation. However, although expressions of different Wnts were seen in the epithelium, Wnt/β-catenin activity was detected only in the mesenchyme. Consistent with this, *Lef1* and *Tcf1*, Wnt signalling mediators, were missing from the epithelium. Nonetheless, Wnts do not signal only through Wnt/β-catenin pathway, but several non-canonical pathways also exist. Some of the analysed ligands can activate non-canonical pathways, resulting in a more complex structure for the signalling pathway as well as complicating the analyses. Thus, the reporter lines, which indicate only active Wnt/β-catenin signalling, might not reveal all active Wnt signalling in the incisor. For instance, *Wnt5a*, often associated as a non-canonical Wnt ligand, is intensely expressed in the mesenchyme next to the lingual cervical loop, suggesting that it might have a role in the negative regulation of epithelial stem cells.

Wnt reporter activity overlapped and was intense in the odontoblasts in all the three reporter lines. *Wnt6* and *Wnt10a* are intensely expressed in the odontoblasts and epithelium aligning the odontoblasts, and epithelium is known to regulate odontoblast differentiation (Thesleff et al., 2001), suggesting that these genes affects the regulation of odontoblast. Wnt10a has also been proposed to participate in odontoblast differentiation in the molars (Yamashiro et al., 2007). However, stabilisation of β-catenin in the mesenchyme leads to the disruption of ameloblast and odontoblast differentiation, suggesting that Wnt/β-catenin signalling has to be strictly balanced during cellular differentiation (Chen et al., 2009). Recently heterozygotes of the same *Axin2*$^{LacZ/LacZ}$ mouse line we analysed were also used to study active Wnt/β-catenin signalling in the post natal tooth. Reporter activity was detected in the odontoblasts before terminal differentiation and in the developing roots (Lohi et al., 2009). Active Wnt/β-catenin might be necessary only during certain stages of odontoblast differentiation and constant activation might lead to disruption of the cells. In the epithelium reporter activity was seen only in the enamel free zones, in the tips of both molars and incisor, and on the lingual side of the incisor (Lohi et al., 2009). These results are in line with our results in analysing the reporter activity of *Axin2*$^{LacZ/LacZ}$ in the embryonic incisor where the ameloblast were devoid of active Wnt/β-catenin signalling (data not shown). Nevertheless weak expression of *Axin2* mRNA was detected in the preameloblasts and ameloblasts. Wnt/β-catenin signalling may have a role in the inhibition of ameloblast differentiation as indicated by intense expression of *Axin2* in the lingual epithelium. The weak *Axin2* mRNA level detected on the labial side could be negatively regulated or might not be intense enough to inhibit ameloblast differentiation.

Lack of active Wnt signalling in the presumptive epithelial stem cells in the labial cervical loop of the incisor was unexpected. Only *Axin2* expression suggested Wnt signalling in the epithelium. *Axin2* was expressed in the preameloblasts and ameloblasts, but the level of expression was low. In the lingual cervical loop of the incisor, where ameloblast differentiation is inhibited, *Axin2* expression was intense. This suggests that Wnt signalling participates in the inhibition of epithelial stem cell proliferation. In hair follicle stem cells Wnt/β-catenin signalling is weak but its upregulation in the transit amplifying cells suggesting that β-catenin stabilization enhances the transition from the stem cells to the transit amplifying cells in the hair follicle (Lowry et al., 2005).

Overexpression of *Wnt3* in the epithelium under *K14* promoter causes progressive loss of ameloblasts postnatally (Millar et al., 2003). Progressive loss of ameloblasts might be due to depletion of stem cells and/or inhibition of stem cell proliferation and differentiation. Our results suggest that Wnt signalling is associated with negative regulation of stem cells and absence of ameloblasts. It is not possible to conclude which stage in ameloblast differentiation was affected in the ameloblasts of *K14-Wnt3* incisor.

The exact location of epithelial stem cells in the cervical loop is still unsolved. The current knowledge of the stem cells comes from several different experiments which nonetheless have not solved exactly which cells are the stem cells in the cervical loop. Lgr5 is a stem cell marker in the intestine and hair follicle, which have the stem cell niches morphologically

resembling the incisor niche (Thesleff and Tummers, 2009). We detected *Lgr5* expression in the cervical loop, within the restricted population of epithelial cells, suggesting that they might be stem cells. However, we did not find any Wnt activity in the proximity of Lgr5 positive cells, suggesting that different mechanisms regulate Lgr5 in the incisor compared to the intestine and hair follicle. Nevertheless, the expression pattern suggests that Lgr5 in the incisor indicates the location of stem cells and that the function of Lgr5 might be conserved in different epithelial stem cells. *Lgr5-/-* mice die at birth (Morita et al., 2004), thus the phenotype of adult *Lgr5-/-* and the adult stem cells is uncharacterised.

Our results suggest that Wnt/β-catenin signalling does not directly regulate the epithelial stem cells of the incisor. However, the expression patterns of Wnt pathway genes together with the analysis of reporter activity indicate that Wnt signalling is active in the mesenchyme surrounding or adjacent to the cervical loops, especially the lingual cervical loop, suggesting that Wnts may have an indirect role in the regulation of cervical loop epithelial cells. The more prominent activity detected on the lingual side suggests that mesenchymal Wnt/β-catenin signalling might have an inhibitory role in epithelial stem cell maintenance.

CONCLUDING REMARKS

Changes in the balance of signalling regulating the development can cause prominent changes in developing tissues and organs. This study showed that changes in the activity of a negative regulator, Fst, resulted in major changes in the development of tooth, including abnormal crown morphology, alterations in enamel patterning and the capacity of teeth to grow and renew.

The different dental characters are flexible and they are regulated by repetitive use of signalling molecules. In mammals, the complexity of dentition has expanded as changes in morphology of tooth rather than as increased number of teeth (Jernvall and Salazar-Ciudad, 2007). Tooth shape show extending morphological diversity in mammals and especially in the molars the characteristics of cusp patterning are varied. The formation of cusps inducing enamel knots is regulated by a competition between activator and inhibitor factors, and alterations in positioning enamel knots can lead to drastic changes in the crown patterning (Salazar-Ciudad and Jernvall, 2002). When the balance of Fst signalling was altered either by loss or gain of function, the molar cusp pattern became aberrant. These results suggest that Fst modulates enamel knot formation and thereby crown morphogenesis, and that this regulation occurs through inhibition of Activin and BMP signalling. Hence, this is another example that the balance of the regulators must be strictly fine-tuned because subtle modifications can cause major changes in the crown patterning.

We identified the molecular mechanisms responsible for asymmetric enamel patterning of continuously growing incisor. Ameloblast differentiation and enamel formation are altered when signalling of two TGFβ family members, BMP and Activin, are modulated by Fst. The reciprocal interaction occurs between three tissue layers, follicle and papilla mesenchyme and dental epithelium between them. We demonstrated that BMP4 regulates differentiation of epithelial stem cells into ameloblasts, which are responsible for enamel formation. The restricted enamel deposition on labial side resulted from the asymmetric expression of *Fst*, which is regulated by Activin, and indicated that Fst is a negative regulator of ameloblast differentiation. On the lingual side Fst inhibits BMP4 and ameloblast differentiation, but on the labial side inhibitory signal is absent from the zone of differentiating preameloblasts and ameloblasts, thus BMP4 is able to induce differentiation. These results indicate the importance of BMP as an inductive signal of ameloblast differentiation. The ameloblast differentiation is suggested to be both non cell-autonomous and cell-autonomous. Shh, together with TGFβ1, may be one of the cell-autonomous signals regulating ameloblasts (Gritli-Linde et al., 2002; Haryuama et al., 2006). Our results uncover a new signalling pathway regulating ameloblast differentiation.

Regenerative medicine has advanced remarkably during the last decade and stem cells are likely to become a valuable tool for it. Still many problems remain, and understanding the precise mechanisms of stem cell maintenance and differentiation, tissue morphogenesis and molecular mechanisms regulating these phenomena need to increase before tissue engineering can become efficient treatment for clinical use. We identified a signalling network regulating epithelial stem cell proliferation in the cervical loops of the incisor. Due to

iterative use of molecules and pathways in different tissues during the development this network may elucidate characteristics of other regulatory networks. Our results indicate that a complex signalling interaction between Activin, BMP, FGF and Fst controls the stem cells. FGF3 induces epithelial stem cell proliferation and BMP4 inhibits *Fgf3* expression. Activin can inhibit the repressive effects of BMP4, but it only occurs on the labial side because asymmetrically expressed *Fst* antagonises Activin signalling, and thus limits the number of lingual stem cells.

Although stem cells have been under vigorous research they have remained elusive in many tissues due to the absence of tools of recognition. Also in the mouse incisors, markers of stem cells are still lacking. Studies concerning stem cells in epidermal organs have been focusing on bulge in the hair follicle and in crypt of the intestine, and several stem cell markers for these niches have been identified, including Lgr5 (Barker et al., 2007; Jaks et al., 2008). The epithelial stem cell niche in the mouse incisor shares some resemblance with these two niches, but whether the *Lgr5* expressing cells which we detected in the incisor are stem cells or not remains to be further analysed. The exact location of incisor stem cells is still debatable, although the first evidences of the stem cell niche location in the cervical loop were achieved already a decade ago (Harada et al., 1999). One challenge for clinical use of stem cells has also been how to obtain the cells. However, recently a method was reported to reprogram cells into pluripotent cells (iPS), indistinguishable from embryonic stem cells, thus giving a possibility to use patient´s own cells for tissue engineering (Takahashi and Yamanaka, 2006; Takahashi et al., 2007).

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